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From: Gabel, Gailene
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Please provide a copy of the following literature ASAP:

1) Rubin et al., New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. Journal of Vascular Surgery, (1992) 15/4 (683-692).

2) Armstrong et al., Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection), THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.

3) ELY et al., PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA, CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904.

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5) Lee et al., In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency. BLOOD, (1979 Mar) 53 (3) 465-71.

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Thanks a bunch,
Gailene R. Gabel
7B15
305-0807

Released Adenosine Diphosphate Stabilizes Thrombin-Induced Human Platelet Aggregates

By Marco Cattaneo, Maria T. Canciani, Anna Lecchi, Raelene L. Kinlough-Rathbone, Marian A. Packham, Pier Mannuccio Mannucci, and J. Fraser Mustard

Normal human platelets aggregated by thrombin undergo the release reaction and are not readily deaggregated by the combination of inhibitors hirudin, chymotrypsin, and prostaglandin E_1 (PGE_1). In contrast, thrombin-induced aggregates of platelets from patients with delta-storage pool deficiency (δ-SPD), which lack releasable nucleotides, are readily deaggregated by the same combination of inhibitors. The ease with which δ-SPD platelets are deaggregated is caused by the lack of stabilizing effects of released ADP, since: (1) exogenous adenosine diphosphate (ADP) (10 μmol/L), but not serotonin (2 μmol/L), abolishes the ability of these inhibitors to deaggregate δ-SPD platelets; (2) thrombin-induced aggregates of platelets from a patient (V.R.) (whose platelets have a severe, selective impairment of sensitivity to ADP, but normal amounts of releasable nucleotides) can be readily deaggregated, and addition of ADP does not stabilize the platelet aggregates; (3) apyrase or creatine phosphate (CP)/creatine phosphokinase (CPK), added before thrombin, make control platelets more easily deaggregated by hirudin, chymotrypsin, and PGE_1, and do not change the deaggregation response of δ-SPD platelets and of V.R.'s platelets. Thrombin-induced aggregation and release of β-thromboglobulin in control, δ-SPD, and in V.R.'s platelets was similar and not inhibited by apyrase or CP/CPK. The stabilizing effect of ADP on platelet aggregates is specific, since epinephrine in the presence of apyrase to remove traces of released ADP does not stabilize the aggregates of control, δ-SPD, or of V.R.'s platelets. Because epinephrine increases fibrinogen binding to thrombin-stimulated platelets to a greater extent than ADP, but does not stabilize the aggregates, it is unlikely that the additional fibrinogen binding sites induced by ADP have a major role in inhibiting deaggregation by the combination of inhibitors.

WHEN HUMAN PLATELETS are aggregated under conditions in which extensive release of granule contents occurs (eg, when they are aggregated by thrombin or when they are aggregated by ADP at micromolar concentrations of Ca^{2+}), they do not deaggregate readily,[1-3] even though combinations of inhibitors, added at the peak of platelet aggregation, dissociate most of the fibrinogen that became associated with the platelets during aggregation.[1]

Experiments with platelets from afibrinogenemic patients showed that when these platelets, which lack fibrinogen in their granules, are aggregated by thrombin, deaggregation by combination of inhibitors does not occur more readily than deaggregation of normal platelets.[4] This observation indicates that fibrinogen may not play a major role in stabilizing thrombin-induced platelet aggregates. Further support for this conclusion comes from the morphometric studies of Suzuki et al[5] showing that in platelet aggregates induced with thrombin there were large areas where platelets were in close contact with little or no fibrinogen between them. Therefore, substances other than fibrinogen that are released from platelets granules stabilize platelet aggregates that have undergone the release reaction.

To examine the role of platelet δ granule contents, particularly of adenosine diphosphate (ADP) in stabilizing thrombin-induced platelet aggregates, aggregation and deaggregation were studied with platelets from patients with delta-storage pool deficiency (δ-SPD).[6,7] Another way of studying the effect of released ADP in the stabilization of platelet aggregates was offered by the availability of a patient described previously,[8,9] whose platelets show a selective congenital impairment of aggregation and fibrinogen binding induced by ADP. With both these types of abnormal platelets we have also investigated whether epinephrine can stabilize thrombin-induced aggregates, and explored the enhancing effect of these agonists on fibrinogen binding during thrombin-induced aggregation. Results from this study indicate that released ADP stabilizes the thrombin-induced human platelet aggregates through a specific mechanism that is independent of the binding of fibrinogen to the platelet glycoprotein IIb/IIIa complex.

MATERIALS AND METHODS

Materials. Carrier-free sodium iodide (^{125}I) was from Amersham International (Buckinghamshire, UK). Apyrase was prepared from potatoes by the method of Molnar and Lorand.[10] The activity of this preparation was such that the enzyme (1 μL/mL) converted 0.25 μmol/L adenosine triphosphate (ATP) to adenosine monophosphate (AMP) within 120 seconds at 37°C. Bovine serum albumin was from Calbiochem, La Jolla, CA. Chymotrypsin, ADP, prostaglandin E_1 (PGE_1), thrombin, serotonin (5-hydroxytryptamine creatinine sulfate complex), epinephrine, creatine phosphate (CP), and creatine phosphokinase (CPK) were from Sigma, St Louis, MO. Hirudin was from Pentapharma (Basel, Switzerland). PGE_1 was prepared as previously described.[11] All other solutions were dissolved in Tyrode buffer.

Preparation of platelet-rich plasma (PRP) and of washed platelet suspensions. Citrated PRP was prepared as described.[12] Platelets

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were washed according to the method described by Mustard et al,[13] and suspended in Tyrode buffer containing 0.35% albumin, 0.1% dextrose, and apyrase (1 μL/mL). Apyrase was omitted from the final platelet suspensions in which thrombin-induced release of adenine nucleotides was measured. The final platelet count was adjusted to 5×10^{11}/L.

Platelet aggregation and release of β-thromboglubulin (BTG). Platelet aggregation was measured in an aggregometer as described previously.[12] The release of BTG was measured by radioimmunoassay (Amersham, UK) in the supernatant of samples obtained by centrifugation in an Eppendorf microcentrifuge (Eppendorf, West Germany) for 120 seconds.

Platelet adenine nucleotide content and release. Platelet ADP and ATP content was measured by a firefly-luciferase method on ethanol:0.1 mol/L EDTA extracts of unstimulated samples of PRP.[14] Released ADP and ATP were measured on ethanol:EDTA extracts of supernatants of washed platelet suspensions incubated at 37°C for 3 minutes with different concentrations of thrombin in the presence of 1.4 mmol/L EDTA.[15] The firefly-luciferase reagent was from LKB-Wallace (Turku, Finland).

Platelet BTG and platelet factor 4 (PF_4). The platelet concentrations of BTG and PF_4 were measured by radioimmunoassay of 1% Triton X-100 (Sigma, St Louis, MO) lysates of PRP samples. The commercially available radioimmunoassay kit from Abbott Laboratories (Chicago, IL) was used to measure PF_4 concentrations.

Iodination of fibrinogen. Human fibrinogen (Grade L, Ab Kabi, Stockholm, Sweden) was pretreated with diisopropylfluorophosphate (Sigma) to inhibit serine proteases,[16] further purified according to the method of Lawrie et al,[17] and labeled with ^{125}I by the method of MacFarlane,[18] to a specific activity of 5,000 cpm/μg.

^{125}I-fibrinogen binding to platelets. Thrombin (0.25 U/mL) was added to 200 μL of washed platelets. In some samples apyrase was added 15 seconds before thrombin. In experiments in which ADP or epineprhine was used in combination with thrombin, the agonist was added 5 seconds after thrombin. After 3 minutes of incubation at 37°C without stirring, hirudin (1.25 U/mL) was added to block further effects of thrombin, followed immediately by ^{125}I-fibrinogen (final concentration of 50 μg/mL). After incubation of the mixture (final volume 250 μL) with ^{125}I-fibrinogen at 37°C without stirring for 15 minutes, platelet-bound ligand was separated from free ligand by centrifugation through 20% sucrose in Tyrode buffer. The radioactivity in the platelet pellet was measured in a gamma counter (LKB 1260, Bromma, Sweden). The specific binding of ^{125}I-fibrinogen was calculated after subtracting the radioactivity associated with unstimulated platelets.

Patients. Patients P.B. (25-year-old man), M.B. (23-year-old man, brother of P.B.), and M.R.C. (33-year-old woman) have a lifelong history of easy bruising, mildly prolonged bleeding times, and normal platelet counts and coagulation tests. Platelet aggregation in citrated PRP was typical of patients with defects of the release reaction,[6,7] with monophasic aggregation to epinephrine (5 μmol/L), ADP (2 μmol/L) or PAF-acether (0.2 μmol/L), and impaired aggregation in response to 1 μg/mL collagen (0% to 40% increase in light transmission, normal range: 49% to 71%) and 0.5 mmol/L arachidonic acid (25% to 45% normal range: 60% to 74%). Measurement of platelet ADP, ATP, PF_4, and BTG (Table 1) and of releasable ADP and ATP (Table 2) showed that the patients were affected by δ-SPD. Patient V.R. is a 50-year-old man with a lifelong history of easy bruising, prolonged bleeding time, and normal platelet count and coagulation tests. Platelet function studies showed a severe, selective impairment of platelet aggregation and fibrinogen binding induced by ADP[8,9]; epinephrine induced a normal primary wave of aggregation of V.R.'s platelets in citrated plasma; the platelet α granule contents and total adenine nucleotides were within the normal range (Table 1). All the patients gave informed consent to the study.

RESULTS

Platelet release reaction, aggregation, and deaggregation. Suspensions of platelets prepared from the blood of the three patients with δ-SPD and of patient V.R. aggregated and released BTG to the same extent as control platelets on stimulation with thrombin. (0.2 to 5 U/mL) (in Fig 1 through 3 the percent release of BTG induced by 0.25 or 1 U/mL thrombin is shown). With concentrations of thrombin lower than 0.2 U/mL, the percent release of BTG from δ-SPD platelets was lower than that from control platelets (not shown). Therefore, studies on the effects of inhibitors on the stability of thrombin-induced platelet aggregates were done with concentrations of thrombin higher than 0.2 U/mL. The amount of ATP and ADP released from δ-SPD platelets stimulated with any concentration of thrombin tested was much less than from control platelets, whereas platelets of patient V.R. released normal amounts of ATP and ADP (Table 2).

The addition of hirudin (1.25 to 5 U/mL, added to block further effects of thrombin) and of Tyrode buffer to the platelet suspensions 2 minutes after stimulation with thrombin (0.25 to 1 U/mL) did not cause the aggregates of control, δ-SPD and V.R.'s platelets to deaggregate (Fig 1A). When PGE_1 (10 μmol/L) plus chymotrypsin (10 U/mL) was added instead of Tyrode buffer to control suspensions, platelets did not deaggregate within 6 minutes after the addition of thrombin (Fig 1B). When platelet aggregation was monitored for longer than 6 minutes, the aggregates of normal

Table 1. Platelet Contents of ADP, ATP, BTG, and PF_4 in PRP Samples of Patients With δ-SPD, of Patient V.R., and of Control Subjects

Subjects	n	ATP (μmoles/10^{11} plts)	ADP (μmoles/10^{11} plts)	ATP/ADP	BTG (ng/10^8 plts)	PF_4 (ng/10^8 plts)
δ-SPD*						
M.B.	2	4.5	0.74	6.1	127	19
P.B.	2	4.6	0.81	5.7	140	31
M.R.C.	2	4.7	0.20	23.1	131	17
Defective response to ADP*						
V.R.	2	4.3	2.3	1.9	134	22
Control (pooled)	22	5.8 ± 0.8	2.8 ± 0.5	2.1 ± 0.3	86 ± 47	23 ± 14

Mean ± SD.

Abbreviation: plts, platelets.

*Mean values of independent determinations on two different samples.

Table 2. Thrombin-Induced Release of Platelet ATP and ADP in Patients With δ-SPD, in Patient V.R., and in Five Control Subjects

Subjects	Thrombin (0.25 U/mL)		Thrombin (1 U/mL)		Thrombin (5 U/mL)	
	ATP	ADP	ATP	ADP	ATP	ADP
δ-SPD						
M.B.	0.2	0.2	0.3	0.33	0.33	0.4
P.B.	0.6	0.64	0.6	0.67	0.7	0.7
M.R.C.	0.2	0.14	0.21	0.12	0.18	0.13
Defective response to ADP						
V.R.	1.4	1.3	2.3	2.0	2.0	2.7
Control (pooled)	1.9 ± 1.08	2.1 ± 0.87	2.26 ± 0.55	2.7 ± 0.32	2.17 ± 0.48	2.8 ± 0.28

Mean values ± SD (μmoles/10^{11} platelets) of duplicate determinations on washed platelet suspensions (platelet count 5 × 10^{11}/L).

platelets began to deaggregate slowly at 7 to 8 minutes after the addition of thrombin, but deaggregation was never complete by 10 minutes. In contrast, the same combination of hirudin, PGE_1, and chymotrypsin caused the aggregates of δ-SPD platelets and of V.R.'s platelets to deaggregate almost completely within 6 minutes (Fig 1B). Higher concentrations of thrombin (up to 5 U/mL) induced aggregates of δ-SPD platelets or V.R.'s platelets that could not be easily deaggregated by the same combination of inhibitors (not shown).

Effects of exogenous ADP or of ADP scavengers on the deaggregation of δ-SPD and of V.R.'s platelets. The addition of ADP (10 μmol/L) to the platelet suspensions 5 seconds after the stimulation with thrombin (0.25 U/mL) abolished the ability of the combination of hirudin, PGE_1, and chymotrypsin to deaggregate the δ-SPD platelets (Fig 2, A and B). The effect of exogenous ADP on deaggregation of δ-SPD platelets was dose-dependent, being maximal at 5 μmol/L in patient P.B. and at 10 μmol/L in patients M.B. and M.R.C. (not shown). Exogenous ADP (10 μmol/L), however, did not affect the ease with which V.R.'s platelets aggregated by thrombin could be deaggregated by the same combination of inhibitors (Fig 2, A and B). Thrombin-induced release of BTG from δ-SPD, V.R.'s, or control platelets was not affected by exogenous ADP.

The addition of apyrase (20 μL/mL) or of CP (4 mmol/L) plus CPK (10 U/mL) to control suspensions 15 seconds before thrombin, made control platelets easily deaggregated by the combination of hirudin, PGE_1, and chymotrypsin (Fig 2C). The addition of the ADP scavengers 15 seconds before thrombin did not affect the deaggregation of δ-SPD or V.R.'s platelets aggregated by thrombin, but reversed the ability of exogenous ADP to stabilize the thrombin-induced aggregates of δ-SPD platelets. The ADP scavengers did not affect the extent of the release of BTG.

Effects of exogenous serotonin on the deaggregation of δ-SPD and of V.R.'s platelets. Serotonin (2 μmol/L) added to the platelet suspensions 5 seconds after thrombin did not affect the release of BTG, nor did it affect the ease with which δ-SPD or V.R.'s platelets were deaggregated by the combination of inhibitors (not shown).

Effects of exogenous epinephrine on the deaggregation of δ-SPD and V.R.'s platelets. Epinephrine (5 μmol/L) added to platelet suspensions 5 seconds after thrombin appeared to induce effects similar to those caused by ADP; ie, it abolished the ability of the combination of inhibitors to deaggregate δ-SPD platelets (Fig 3A). However, because the thrombin-stimulated δ-SPD platelets released small amounts of ADP (Table 2) with which epinephrine could synergize, the experiments with epinephrine were repeated in the presence of apyrase (20 μL/mL) or CP/CPK (4 mmol/L, 10 U/mL). When one of these ADP-degrading systems was present, epinephrine did not stabilize the thrombin-induced aggre-

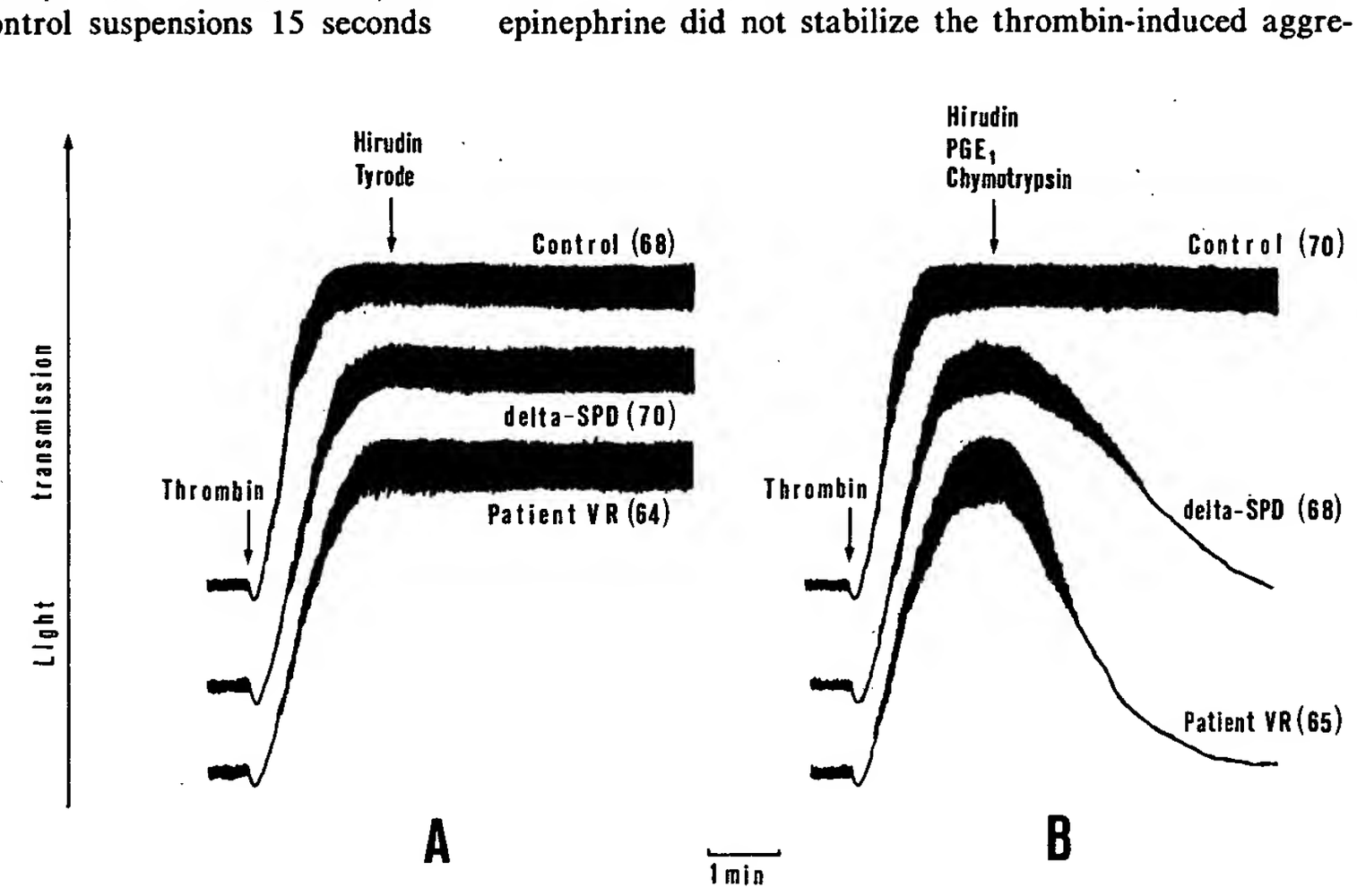

Fig 1. Effects of Tyrode buffer (A) or PGE_1 (10 μmol/L) and chymostrypsin (10 U/mL) (B) on deaggregation of platelets aggregated by thrombin (1 U/mL). The δ-SPD patient was M.B. The highest concentration of thrombin tested that induced labile aggregates of δ-SPD and V.R.'s platelets varied from 0.25 to 1 U/mL. Numbers within brackets indicate the percent release of BTG from platelets. Hirudin (5 U/mL) was added before PGE_1 and chymotrypsin to block further effects of thrombin. Representative of 19 experiments.

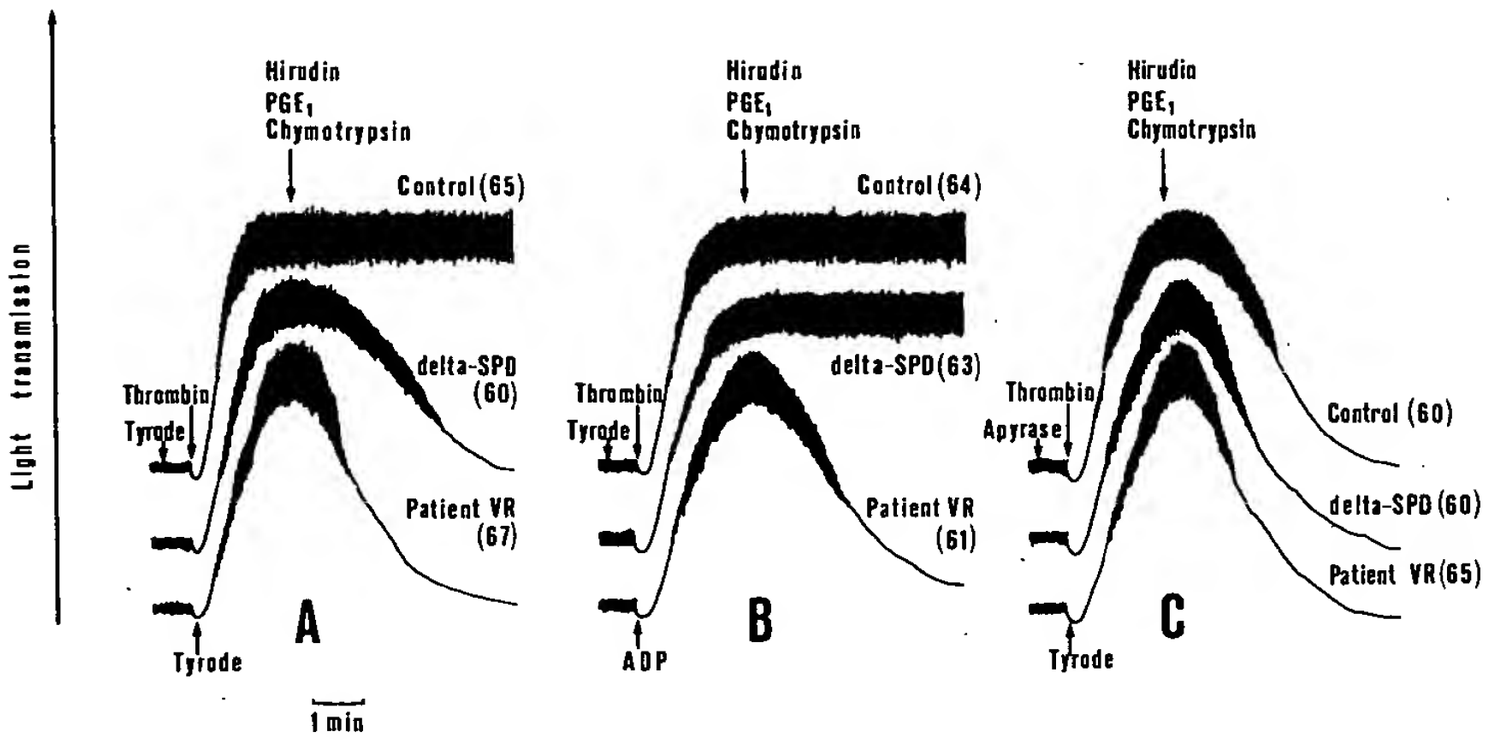

Fig 2. Effects of Tyrode buffer (A), ADP (10 μmol/L, [B]) or apyrase (20 μL/mL, [C]) on deaggregation by PGE_1 (10 μmol/L) and chymotrypsin (10 U/mL) of platelets aggregated by thrombin (0.25 U/mL). The δ-SPD patient was M.R.C. ADP or Tyrode was added to the platelet suspensions 5 seconds after thrombin. Apyrase or Tyrode was added 15 seconds before thrombin. Similar results were obtained with CP/CPK added instead of apyrase. Numbers within brackets indicate the percent release of BTG from platelets. Hirudin (1.25 U/mL) was added before PGE_1 and chymotrypsin to block further effects of thrombin. Representative of 13 experiments.

gates of δ-SPD platelets or of control platelets (Fig 3B), indicating that traces of released ADP were responsible for the effect seen with δ-SPD platelets in Fig 3A. In keeping with this conclusion is the finding that epinephrine had no effect on the stability of V.R.'s platelet aggregates induced by thrombin (Fig 3, A and B).

Thrombin-induced release of BTG from control, δ-SPD, and V.R.'s platelets was not affected by epinephrine, either in the presence or absence of apyrase or CP/CPK.

^{125}I-fibrinogen binding. Platelets from patient M.R.C. with δ-SPD and from patient V.R. (with a defect in responsiveness to ADP) bound considerably less ^{125}I-fibrinogen than normal platelets after stimulation with thrombin (0.25 U/mL) (Fig 4). Apyrase inhibited the thrombin-induced ^{125}I-fibrinogen binding to control platelets, but had little effect on ^{125}I-fibrinogen binding to platelets from these two patients. The addition of ADP (10 μmol/L) 5 seconds after thrombin increased the binding of ^{125}I-fibrinogen to the δ-SPD platelets, but had no effects on ^{125}I-fibrinogen binding to control or to V.R.'s platelets.

The addition of epinephrine (5 μmol/L) 5 seconds after thrombin increased the binding of ^{125}I-fibrinogen to control,

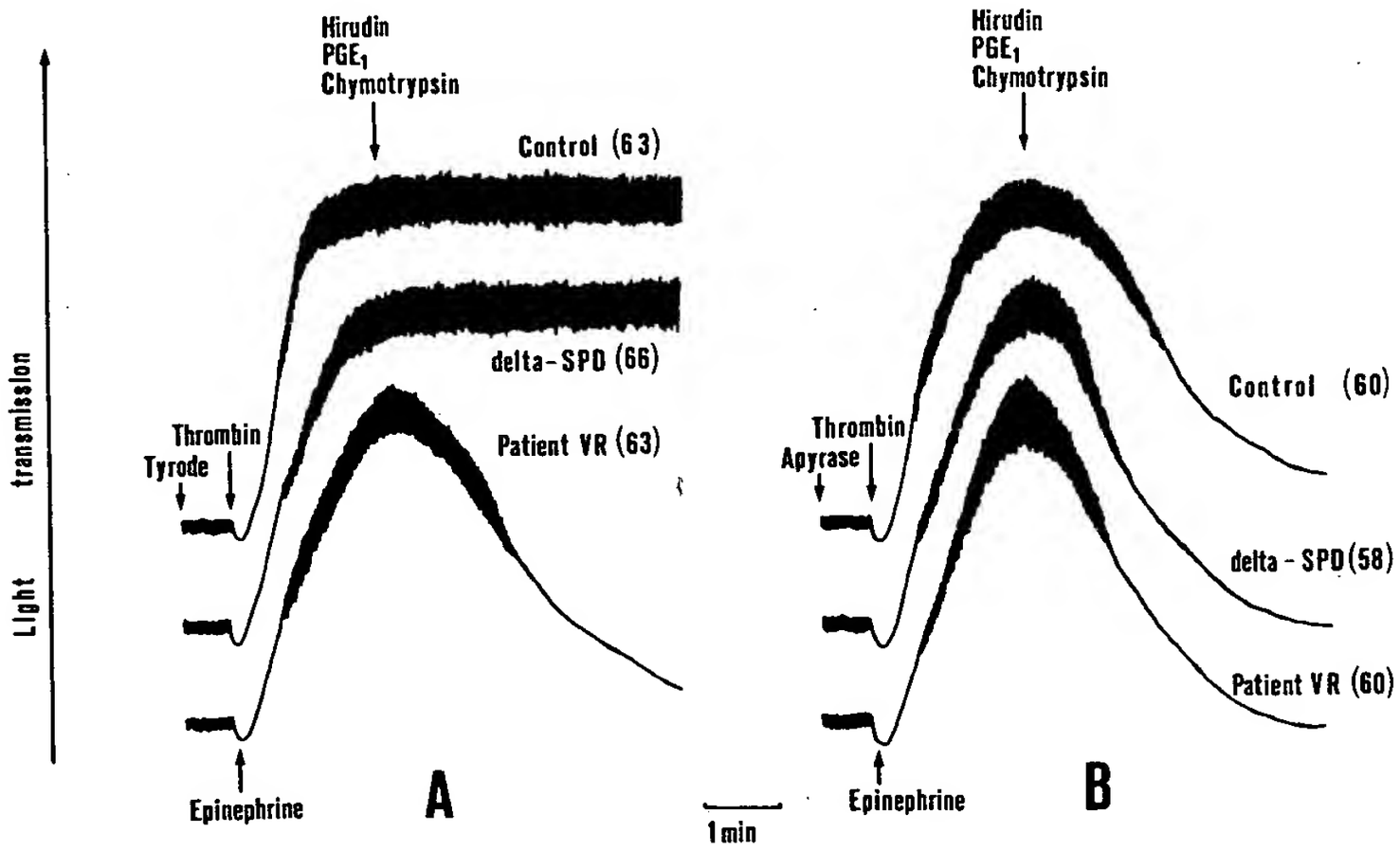

Fig 3. Effects of epinephrine (5 μmol/L, [A]) or of apyrase (20 μL/mL) and epineprhine (5 μmol/L, [B]) on deaggregation by PGE_1 (10 μmol/L) and chymotrypsin (10 U/mL) of platelets aggregated by thrombin (0.25 U/mL). The δ-SPD patient was M.R.C. Apyrase or Tyrode buffer was added 15 seconds before thrombin. Epinephrine was added 5 seconds after thrombin. Similar results were obtained with CP/CPK added instead of apyrase. Numbers within brackets indicate the percent release of BTG from platelets. Hirudin (1.25 U/mL) was added before PGE_1 and chymotrypsin to block further effects of thrombin. Similar results were obtained with platelets from the other two δ-SPD patients studied. Representative of nine experiments.

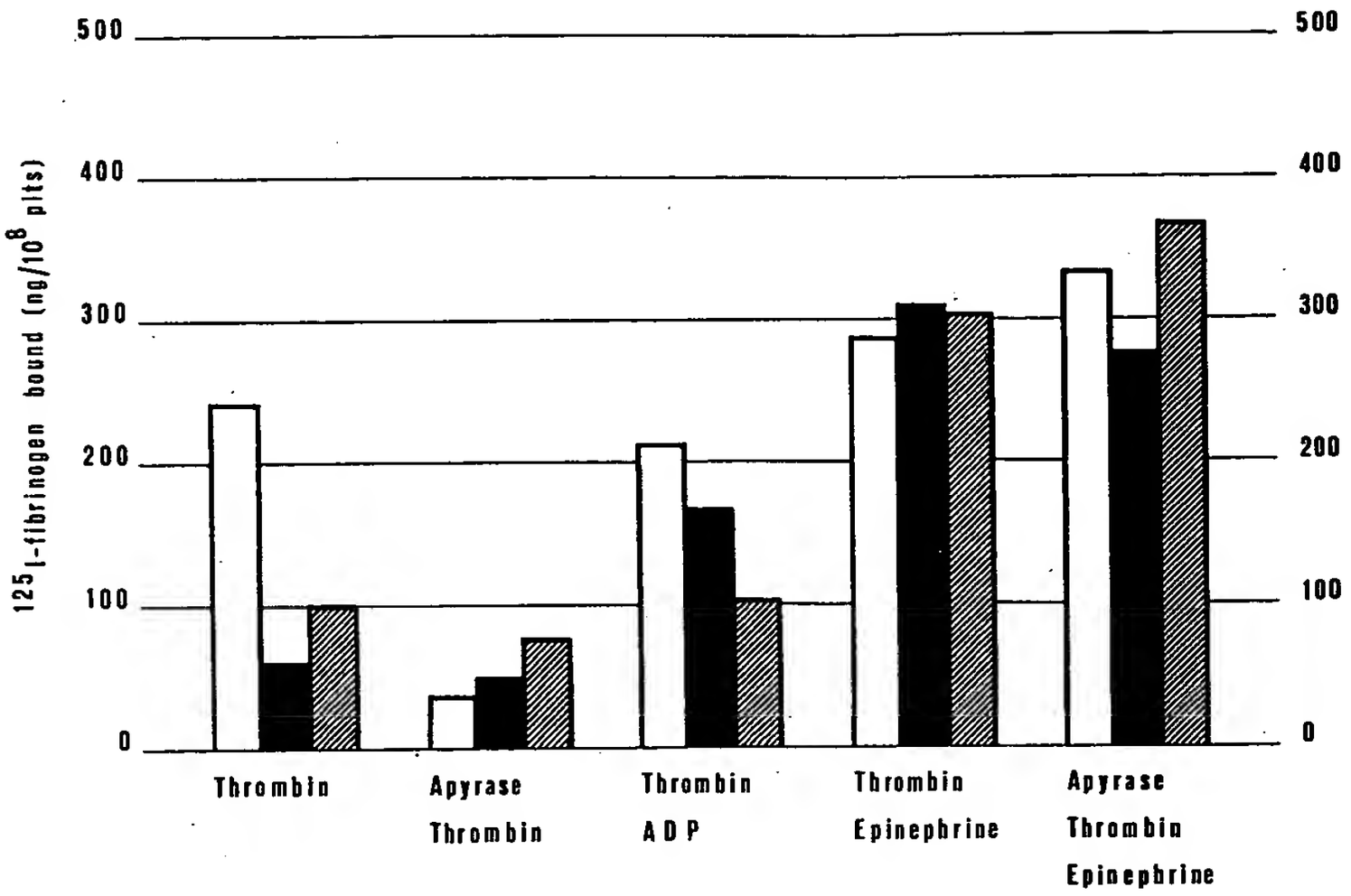

Fig 4. Specific ^{125}I-fibrinogen binding to platelets from control (□), δ-SPD patient (M.R.C., ■), and patient V.R. (▨) induced by thrombin (0.25 U/mL). Each bar represents the mean values of two experiments with triplicate determinations. Apyrase (20 μL/mL) was added 15 seconds before thrombin. ADP (10 μmol/L) or epinephrine (5 μmol/L) was added 5 seconds after thrombin. See Materials and Methods for details.

δ-SPD, and V.R.'s platelets to similar values (Fig 4). The addition of apyrase 15 seconds before thrombin had little effect on this epinephrine-induced increase in ^{125}I-fibrinogen binding.

DISCUSSION

Normal human platelets aggregated by thrombin release the contents of their storage granules. In previous studies[2] we found that platelets aggregated by thrombin were only slightly deaggregated by 6 to 8 minutes when a combination of hirudin, chymotrypsin, and PGE_1 was added after thrombin-induced aggregation had reached its maximum. In this study, three lines of evidence indicate that ADP released from the platelets during thrombin-induced aggregation contributes to the stability of human platelet aggregates: (1) platelets from patients with δ-SPD contain few releasable adenine nucleotides, and after aggregation by thrombin can be readily deaggregated by combinations of inhibitors; exogenously added ADP (but not serotonin) blocks the ability of the combination of inhibitors to deaggregate these platelets. (2) Thrombin-induced aggregates of platelets from a patient (V.R.) with a selective defect of platelet sensitivity to ADP[8,9] can also be easily deaggregated by the combination of inhibitors. Addition of ADP immediately after the addition of thrombin to platelets from this patient does not change the extent to which thrombin-induced platelet aggregates can be deaggregated because these platelets are unresponsive to ADP. (3) The importance of released ADP in stabilizing thrombin-induced platelet aggregates is further supported by the finding that normal platelets are more readily deaggregated by the combination of hirudin, chymotrypsin, and PGE_1 in the presence of ADP scavengers such as apyrase or CP/CPK, which have no effect on the rate of deaggregation of thrombin-induced aggregates of platelets from patients with δ-SPD or from patient V.R. Thus, it is evident that ADP, normally released from platelet-dense granules on stimulation with thrombin, plays a part in stabilizing thrombin-induced platelet aggregates. These results are in accord with the recent conclusion of Maffrand et al[19] from their experiments with fawn-hooded rats, which lack ADP in their platelet-dense granules; these investigators showed that thrombus formation on a silk thread in an arteriovenous shunt was greatly reduced in these animals, and they concluded that ADP plays a major role in thrombogenesis.

The mechanism by which released ADP stabilizes platelet aggregates is unknown. ADP itself does not produce stable aggregates unless the release of granule contents has occurred[1]; therefore, to stablize platelet aggregates, ADP may either require some other granule contents released from platelets or synergize with thrombin. This effect is not related to the known potentiating effect of released ADP on platelet aggregation and the release reaction[20,21] because experiments were performed at concentrations of thrombin that were sufficiently high to induce the same extent of aggregation and release of BTG in platelets from normal controls, δ-SPD patients, and from patient V.R.

The stabilizing effect of ADP that we have observed is specific since neither serotonin or ephinephrine (in the presence of ADP scavengers so that there is no trace of ADP with which epinephrine can synergize[22]) stabilized the thrombin-induced aggregates of δ-SPD platelets. In addition, epinephrine or serotonin did not stabilize thrombin-induced aggregates of V.R.'s platelets, which undergo normal primary phase of aggregation on stimulation by epinephrine in citrated plasma.[23]

The observation that released ADP is not essential to stabilize human platelet aggregates induced by concentrations of thrombin higher than 0.25 to 1 U/mL suggests that high concentrations of thrombin either trigger the same mechanism(s) as released ADP or cause specific platelet alterations that stabilize the aggregates. Bauman et al[3] indicated that extensive secretion per se is not responsible for

the irreversible aggregation of human platelets induced by thrombin, and proposed that the formation of irreversible linkages between platelets involves the specific, time-dependent interaction of thrombin with platelets, released fibrinogen, and possibly one or more substances secreted from platelets.

The present experiments indicate that it is unlikely that the additional fibrinogen binding sites induced by released ADP play an important role in stabilizing thrombin-induced aggregates since epinephrine increased the thrombin-induced fibrinogen binding in the presence of apyrase more strongly than ADP, but epinephrine did not stablize the aggregates of control, δ-SPD, or V.R.'s platelets induced by thrombin in the presence of apyrase. Our earlier findings[4] that washed platelets from patients with afibrinogenemia are no more readily deaggregated by the combination of inhibitors than platelets from control subjects, also indicate that fibrinogen binding does not have a major role in the stabilization of thrombin-induced platelet aggregates.

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In Vitro Platelet Abnormality in Adenosine Deaminase Deficiency and Severe Combined Immunodeficiency

By Choon H. Lee, Susan P. Evans, Maurice C. Rozenberg, Aldo S. Bagnara, John B. Ziegler, and Martin B. Van der Weyden.

The platelets of an infant with severe combined immune deficiency and adenosine deaminase deficiency showed markedly diminished responses to ADP-induced aggregation in vitro. This abnormality was corrected by the addition of purified adenosine deaminase in vitro. Exogenous adenosine added to platelet-rich plasma caused markedly prolonged inhibition of ADP-induced aggregation. This was shown by isotopic studies to be due to slow clearance of adenosine and hence persistence of this nucleoside. Direct assay for adenosine deaminase in plasma and platelet lysates of the patient confirmed the very low activity of this enzyme. Raised cAMP levels were demonstrated in his platelets. The deranged adenosine metabolism and raised cAMP in the platelets of this child with severe combined immunodeficiency may explain the altered response to ADP. Despite the in vitro platelet aggregation abnormality, the patient had no clinical evidence of impaired hemostasis.

THE SYNDROME of severe combined immune deficiency (SCID) with adenosine deaminase (ADA) deficiency was first described in 1972.[1] A 4-mo-old infant with this syndrome was referred to us for diagnosis and management. ADA activity was found to be undetectable in his erythrocytes and serum. He was commenced on a course of red cell transfusion therapy similar to that of Polmar et al.,[2] providing us with sufficient blood to study his platelet function and metabolism. A full report of his clinical course is to be published elsewhere.[3]

ADA converts the purine nucleoside adenosine to inosine. In ADA-negative SCID, the enzyme deficiency has been demonstrated in a wide variety of tissues, including sera, erythrocytes, leukocytes, spleen, liver, and fibroblasts. It is reasonable to expect that the platelets will also lack the enzyme, although this fact has not been specifically recorded. Lack of this enzyme would block a major pathway of adenosine metabolism in platelets[4] and other tissues and could conceivably lead to accumulation of adenosine in vivo. Adenosine is known to inhibit ADP-induced platelet aggregation in vitro[5] and in vivo.[6] This article reports the patient's platelet responses to various aggregating agents in vitro and the ADA activity of his platelets and plasma. We also studied the inhibitory effect on ADP-induced aggregation by exogenous adenosine, and this was correlated with simultaneous

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metabolic study of PRP using [8-^{14}C]-adenosine. The correction of an aggregation abnormality of the patient's platelets to ADP-induced aggregation by highly purified ADA was then demonstrated. Adenine nucleotide levels in his platelets were measured by high-pressure liquid chromatography and cAMP levels by radioimmunoassay, as these parameters could have significant implications for the inhibitory role of adenosine. During the preparation of this manuscript, the first report of a platelet aggregation defect in ADA-negative SCID was published.[7]

MATERIALS AND METHODS

All the studies on the patient reported in this article were carried out just prior to each exchange transfusion; in all instances circulating erythrocyte ADA activity was less than 15% of the mean value for normal subjects. Blood was collected by clean venipuncture into plastic tubes containing 0.10 volume of 3.8% sodium citrate. Platelet-rich and platelet-poor plasmas were then obtained by centrifugation at 400 *g* and 1000 *g*, respectively, for 10 min at 20°C. The platelet-rich plasma (PRP) was then adjusted to a count of 200–250 X $10^3/\mu l$ for aggregometric studies. All studies were completed within 2½ hr of blood collection. Aggregating agent (0.1 ml) was added to PRP (0.9 ml) in the cuvette of an Eel titrator kept at 37°C, and the change in optical density was recorded on a Servoscribe RE 511.20. Both the rate of the aggregation response %Δ O.D./min and the maximum aggregation achieved %Δ O.D. were calculated. Aggregating agents tested (final concentrations given) included collagen (1 mg/ml), ristocetin (1.5 mg/ml), bovine fibrinogen (1.2 mg/ml), adrenalin (50 μg/ml), and ADP (1.25-μM, 2.50-μM, and 5.0-μM) (2.5 μg/ml = 5-μM).

Inhibitory effects of exogenous adenosine were studied by incubating PRP with adenosine (12.5-μM) at 37°C in stoppered plastic tubes. At timed intervals, continuing up to 90 min, ADP-induced aggregation was measured (ADP = 5.0-μM) as above. Inhibition was measured by comparing the aggregation response with that obtained with PRP incubated with the same small volume of Owren's buffer for a similar time period (Fig. 1A). At the same time the metabolism of adenosine in PRP was studied using [8-^{14}C]-adenosine, also at 12.5-μM, under the same conditions. Samples were deproteinized at timed intervals with 0.5-*M* perchloric acid at 0°C. This was subsequently neutralized with 4.5-*M* KOH/1.0-*M* $KHCO_3$, and 5 or 10 μl of supernatant were used for chromatographic analysis. Resolution of purine compounds was obtained either by two-dimensional paper chromatography[4] or by thin-layer chromatography on PEI-cellulose (Machery-Nagel).[8] Spots were visualized under UV light, cut out and placed in 4 ml of scintillation fluid (*p*-terphenyl in toluene or PCS Amersham), and counted with about 55% efficiency in a Packard Tricarb beta scintillation counter.

The effect of exogenous purified ADA (Sigma type III) on ADP-induced platelet aggregation was also studied. PRP was incubated at 37°C with ADA (at a final activity of 2 IU/ml) for 10 min, and ADP-induced aggregation was compared with that of PRP incubated with Owren's buffer for a similar time. All three concentrations of ADP were employed.

Platelet lysates were prepared from PRP (anticoagulated with 0.075 volume of 0.077-*M* sodium EDTA, pH 7.4) by washing a known number of platelets (counted by a Coulter thrombocounter) twice with a mixture of 0.154-*M* NaCl, 0.154-*M* TRIS (pH 7.4), and 0.077-*M* sodium EDTA (90:8:2 by volume). The platelet button was freeze-thawed three times in dry ice/acetone, and a small volume of TRIS saline (20-m*M* TRIS, pH 7.4) was added to reconstitute a crude platelet lysate. ADA activity in platelet lysates was measured in a standard assay. The ADA assay mixture, kept at 37°C, consisted of 50 μl of platelet lysate (approximately 1×10^8 platelets) and 25 μl of 1-m*M* [8-^{14}C]–adenosine (10 mCi/mmole). The reaction was stopped after 15 min by the addition of 25 μl of 3-*M* $HClO_4$ at 0°C. The contents were then neutralized with 15 μl of 4.5-*M* KOH/1.0-*M* $KHCO_3$, and chromatographic separation of inosine and hypoxanthine from adenosine was achieved with one-dimensional paper chromatography[4] using only the first solvent or with thin-layer chromatography on PEI-cellulose using 1-butanol:acetic acid:H_2O (5:3:2 by volume). There was no radioactivity in nucleotide spots. Counting procedures were as described above, the sum of the radioactivity in the inosine and hypoxanthine spots being taken as product of the ADA reaction. Activity was expressed as micromoles per hour per 10^{11} platelets.

Plasma ADA activity was determined as described above for platelet lysates, but using 50 μl of citrated plasma. In calculating the plasma ADA activity, the diluting effect of citrate was taken into consideration, and the results were expressed as micromoles per minute per liter of plasma.

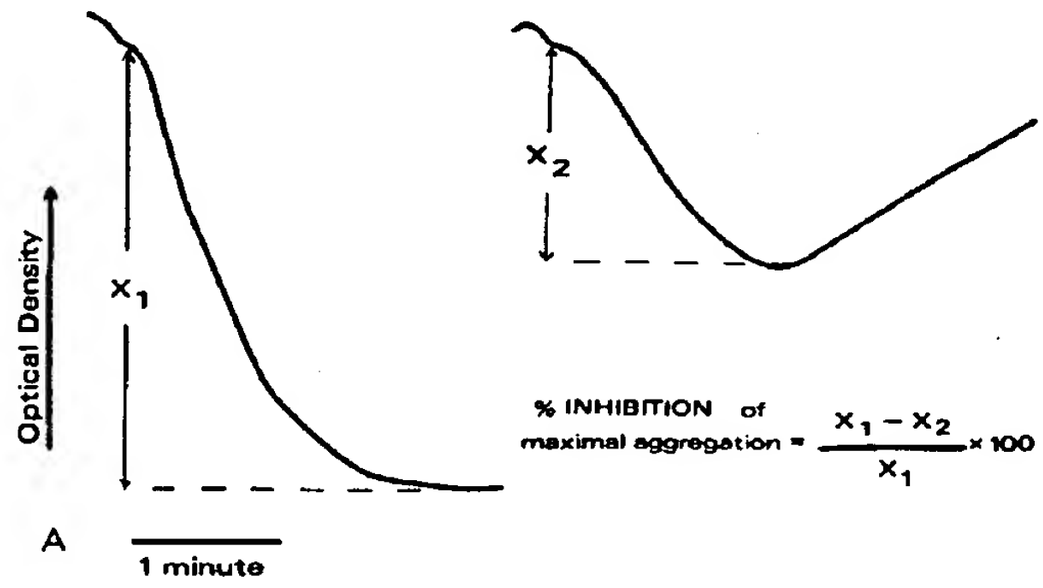

Fig. 1. (A) Measurement of inhibition of ADP-induced platelet aggregation by exogenous adenosine. Curve on left represents response of PRP (incubated with buffer) to ADP (5-μM); curve on right represents response of PRP (incubated with 12.5-μM adenosine for a similar time interval) to ADP. Percentage inhibition is based on measuring maximal aggregation. Similar calculations can be done for aggregation rates. (B) Curves of recovery of platelet aggregation from adenosine inhibition in 6 normal subjects, the patient, and his mother. The patient was studied on two different occasions (adenosine 12.5-μM, ADP 5.0-μM).

Adenine nucleotide levels in platelets were measured in perchloric acid extracts of platelets by high-pressure liquid chromatography using an Altex model 110 system fitted with a Partisil-10 SAX anion-exchange column. Elution was performed at a flow rate of 2 ml/min using a 20-min linear gradient from 10-mM to 500-mM $NH_4H_2O_4$ (pH 4.0). Adenine nucleotide peaks were detected at 254 nm and were quantified using an Autolab Integrator with peak area calibration curves constructed from standard nucleotide solutions. Cyclic AMP levels in platelets were measured in similar platelet extracts using the cAMP radioimmunoassay kit (Amersham).

RESULTS

Table 1 shows the aggregation responses. There were reduced rates and extents of aggregation with all concentrations of ADP used and essentially normal aggregation behavior with the other agents. Disaggregation occurred even at high levels of ADP (5.0-μM).

Figure 1B shows the rates of recovery from adenosine inhibition in 6 normal subjects, the patient's mother (heterozygote), and the patient. ADP-induced platelet aggregation (ADP = 5.0-μM) returned to normal at between 40 and 90 min with platelets from normal subjects and from the mother. There was virtually no recovery from adenosine inhibition with the patient's platelets studied on two different occasions.

Table 1. Platelet Aggregation Studies in the Patient With ADA-Negative SCID

	Aggregation Rate (%ΔO.D./min)	Maximal Aggregation (%ΔO.D.)
Collagen (1.0 mg/ml)	63	70
	(43–98)*	(49–86)
Ristocetin (1.5 mg/ml)	41	82
	(22–66)	(55–98)
Bovine fibrinogen (1.2 mg/ml)	Normal two-phase aggregation	
Adrenalin (50 μg/ml)	Normal two-phase aggregation	
ADP (1.25-μ*M*)	5	4
	(22–84)	(12–84)
ADP (2.5-μ*M*)	20	18
	(40–108)	(20–80)
ADP (5.0-μ*M*)	36	35
	(53–102)	(42–87)

*Values in parentheses represent range of values obtained in at least 25 normal subjects.

Figure 2 shows the results of ^{14}C tracer studies on the PRP of the patient and of a normal subject. [8-^{14}C]-adenosine was rapidly metabolized with reciprocal accumulation of [8-^{14}C]–inosine and hypoxanthine and some incorporation into nucleotides, as expected in normal PRP.[4] Deamination of adenosine was slow in the patient's PRP, with appreciable incorporation of adenosine into adenine nucleotides. These results indicate that the patient's PRP has exceedingly low ADA activity. This finding is substantiated by direct measurements of ADA activity (Table 2), which showed that there was indeed greatly decreased ADA activity in the patient's plasma and platelet lysates.

ADP-induced aggregation was corrected to within the lower limit of the normal response on addition of exogenous ADA (2 IU/ml) (Fig. 3, Table 3). The corrective effect of ADA on platelet aggregation was abolished when the enzyme was

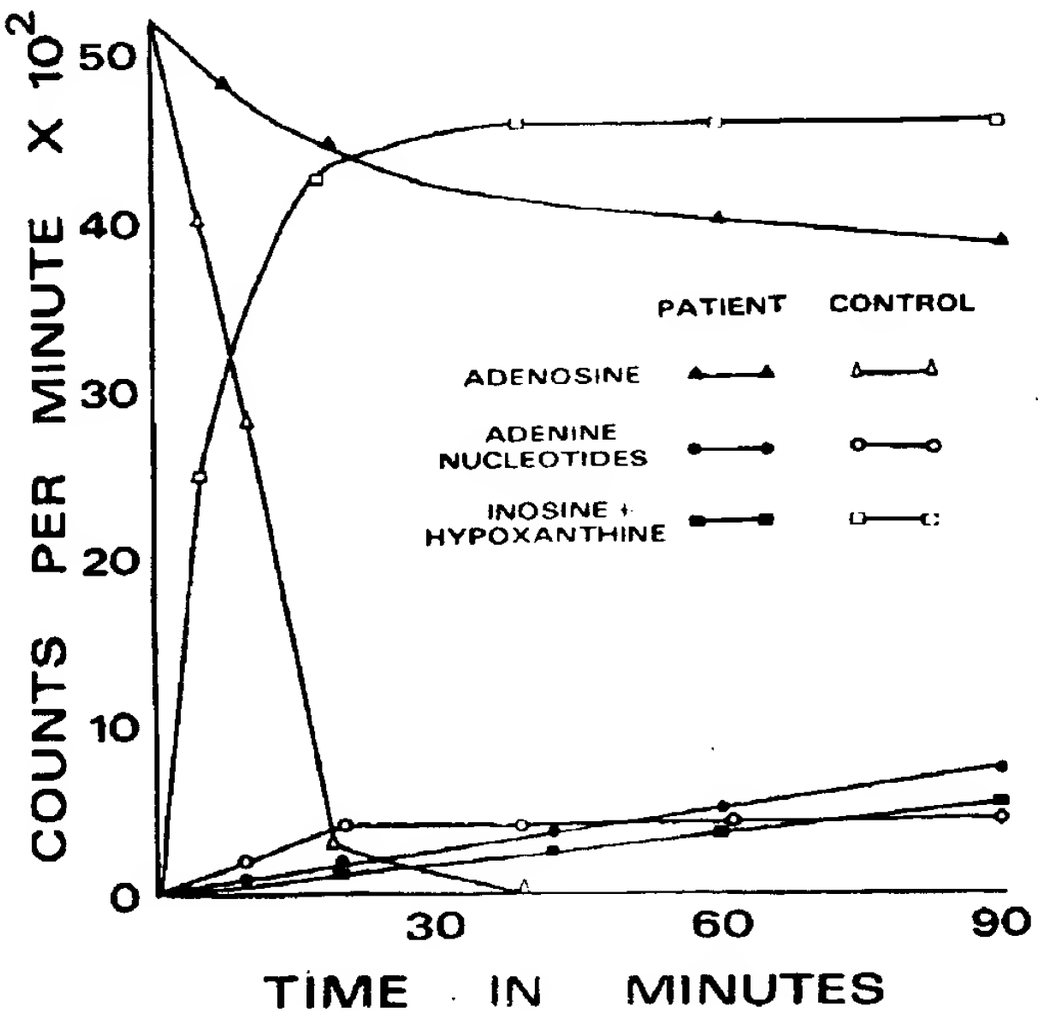

Fig. 2. Metabolism of [8-^{14}C]–adenosine (12.5-μ*M* S.A. 25 mCi/mmole) in PRP of a representative control ADA-positive subject and of the patient with ADA-negative SCID.

Fig. 3. induce all thr 5.0-μ*M*)

Table 2. ADA Activity in Platelet Lysates and Plasma of the Patient With SCID, His Parents, and Normal Controls

Platelet lysates (μmoles/hr/10^{11} platelets)	
Patient	0.1
Mother	8.8
Father	8.4
Controls ($n = 9$)	11.0–16.4
Plasma (μmoles/min/liter)	
Patient	
12/3/77	0
19/5/77	0.36*
20/6/77	0.53*
28/7/77	0.55*
Mother	1.26
Father	1.59
Controls ($n = 11$)	2.33–7.05

*These low plasma levels of ADA activity persist for many weeks after red cell transfusion.

inactivated by prior heating at 80°C for 15 min. Aggregation of normal platelets was unaffected by exogenous ADA.

Nucleotide levels in the patient's normal platelets are shown in Table 4. There was a twofold to threefold increase in cAMP levels in the patient's platelets. However, non-cyclic adenine nucleotide levels were normal. Our values for normal subjects agree well with those reported by others,[9–11] although some authors reported rather high cAMP levels in some normal platelets.[10]

DISCUSSION

We have shown that in one patient with SCID and ADA deficiency the platelets are relatively unresponsive to ADP. Schwartz et al.[7] have reported similar findings. However, they also observed a prolonged lag phase and subnormal aggregation reponses to collagen at a final concentration of 30 μg/ml, in contrast to the normal responses to collagen in our studies. This discrepancy may be explained by the higher concentration of collagen (1 mg/ml) used by us. However, it is pertinent to emphasize here that the failure of our patient to respond to enzyme replacement by exchange transfusion, in contrast to the success of this therapeutic method in the

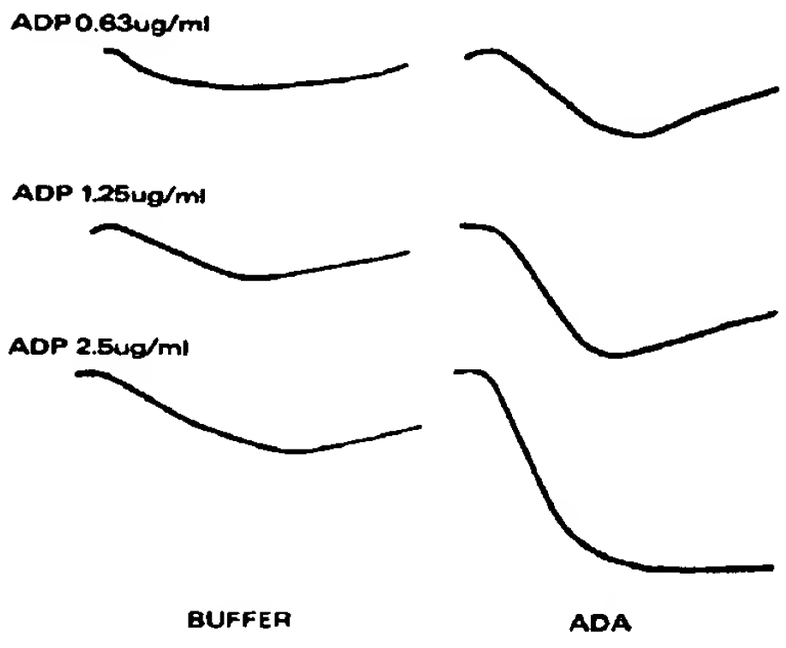

Fig. 3. Correction of the abnormality of ADP-induced platelet aggregation by purified ADA at all three concentrations of ADP (2.5 μg/ml = 5.0-μM).

Table 3. Correction of Platelet Aggregation Abnormality in ADA-Negative SCID by Exogenous Bovine ADA In Vitro

	ADP (1.25-μM)		ADP (2.5-μM)		ADP (5.0-μM)	
	−ADA	+ADA*	−ADA	+ADA	−ADA	+ADA
Aggregation rate (ΔO.D./min)	3 (NR 22–84)	22	5 (NR 40–108)	47	18 (NR 53–102)	57
Maximal aggregation (%ΔO.D.)	3 (NR 12–84)	10	3 (NR 20–80)	20	12 (NR 42–87)	42

*ADA at a final concentration of 2 IU/ml.
NR = normal range.

patient studied by Schwartz et al.,[2,3] may point to some inherent differences in the nature of the enzyme defect in the two cases and may therefore provide a possible alternative explanation for the differences in observations on platelet behavior.

Lack of second-phase platelet aggregation induced by adrenalin in a patient with ADA-negative SCID and in his parents has been reported by Keightley.[12] This abnormality was apparently corrected by incubation with 1-m*M* uridine for 1 hr at room temperature. However, the second wave of adrenalin-induced aggregation can be absent in 10%–20% of normal subjects.[13] Our patient and his mother did not show this abnormality at the concentration of adrenalin employed.

That the phenomenon of decreased platelet responsiveness to ADP in our patient is related to ADA deficiency is suggested by the remarkable correction of the abnormality by exogenous ADA. Agarwal and Parks[9] observed that the addition of coformycin, a potent ADA inhibitor, to normal PRP significantly prolonged the adenosine inhibition of ADP-induced aggregation of those platelets. A similar prolonged inhibition of ADP-induced aggregation of the platelets in our patient was observed; this was related to the slow clearance of adenosine in his PRP, a consequence of low ADA activity in his plasma and platelets. Schwartz et al.,[7] using adenosine at half-molar and equimolar concentrations of ADP, found no further inhibition of the impaired ADP-induced platelet aggregation in their patient. Again, this difference in observation from ours could be due to the higher concentration of adenosine we used (adenosine:ADP at molar ratio of 2.5:1) or to inherent differences in patients.

Adenosine is believed to cause inhibition of platelet aggregation by stimulation of adenyl cyclase, thus raising the level of intracellular cAMP.[14] The increased levels

Table 4. Adenine Nucleotides and cAMP in Platelets of the Patient With ADA-Negative SCID, His Parents, and Controls

	ATP	ADP (μmoles/10^{11} platelets)	AMP	cAMP (nmoles/10^{11} platelets)
Patient	5.0	2.0	0.3	3.5
Controls				
1	5.5	2.8	0.3	1.3
2	8.0	5.5	*	—
3	5.5	3.1	0.4	0.7
4	5.0	3.0	*	—
Mother	—	—	—	0.7

*Levels too low for accurate measurement. Dash indicates not determined.

of cAMP in our patient's platelets would support this hypothesis and imply exposure to adenosine in vivo. Grossly elevated levels of cAMP and ATP have been found in the lymphocytes of ADA-negative SCID patients.[15] However, we were unable to document similar increases in ATP levels in the patient's platelets.

Despite the platelet defect in vitro in this patient, he had no bleeding tendencies. The Ivy puncture bleeding time was 4 min (normal range 4–7 min). The patient of Schwartz et al.[7] also had no bleeding manifestations and had a very marginally prolonged Ivy bleeding time. The significance of the in vitro platelet abnormality is therefore unclear.

ACKNOWLEDGMENT

We wish to thank Professor J. Beveridge for allowing us to study this patient.

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New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts

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Platelet adhesion and aggregation are mediated by fibrinogen via the receptor glycoprotein IIb/IIIa, which recognizes the arginine-glycine-aspartic (RGD) amino-acid sequence. We investigated the ability of 8-guanidino-octanoyl-Asp-Phe (SC-49992), an intravenously infused, stable RGD analogue, to inhibit human platelet function in vitro and to reduce in vivo canine platelet deposition on prosthetic grafts. Human platelet aggregation induced by 10 μmol/L adenosine diphosphate was inhibited in a concentration dependent manner with an ED_{50} of 1 μg/ml of SC-49992. Adenosine diphosphate–induced secretion, which is dependent on fibrinogen occupancy of the glycoprotein IIb/IIIa receptor, was reduced in a concentration dependent manner, also with an ED_{50} of 1 μg/ml. Thrombin-induced secretion, which is independent of fibrinogen binding, was unaffected. Activation-dependent platelet adhesion to fibrinogen substrates was reduced in a concentration-dependent manner by SC-49992. Platelet adhesion to fibronectin substrates was also reduced by the analogue, but to a lesser extent. SC-49992 effectively eluted glycoprotein IIb/IIIa bound to RGD derivatized sepharose. Eight thrombosis-prone dogs had polytetrafluoroethylene femoral artery grafts placed. Dogs received the RGD analogue or a normal saline infusion during their first graft procedure. One week later a second contralateral femoral graft with infusion of the other agent was performed. Aggregometry during RGD analogue infusion demonstrated inhibition of induced aggregation, whereas normal saline infusion had no effect. As measured by the adherence of platelets labeled with indium III 8-guanidino-octanoyl-Asp-Phe reduced platelet deposition on vascular grafts by more than 90% ($p = 0.0006$, log transformed data, paired t test). Histologic examination demonstrated marked reduction or complete elimination of platelet thrombus on the luminal surface of the grafts under drug-treated conditions. Previous attempts to block platelet aggregation have been of limited success. 8-guanidino-octanoyl-Asp-Phe represents a novel class of glycoprotein IIb/IIIa inhibitors, which act at the final common pathway of platelet aggregation. Although the specific role of RGD inhibition in the clinical setting remains undefined, a broad range of platelet-mediated primary and recurrent thromboembolic conditions may potentially benefit from therapeutic intervention with this compound. (J VASC SURG 1992;15:683-92.)

Early occlusion of small diameter synthetic vascular grafts is primarily due to platelet thrombus formation.[1] The formation of platelet thrombus is due to platelet aggregation, which is mediated by the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor.[2] Although normally not expressed on the platelet surface until platelet activation, GPIIb/IIIa exists in extremely large numbers, approximately 50,000 copies per platelet, making it perhaps the most dense receptor for adhesion and aggregation on any cell.[3-5] When activated, this receptor binds several different ligands including fibrinogen, von Willebrand factor, fibronectin, thrombospondin, and perhaps vitronectin.[2-4,6,7] Studies from several laboratories indicate that at physiologic concentrations, fibrinogen is the major ligand bound; fibrinogen's dimeric structure allows it to interact with two platelets simultaneously, leading to aggregation.[2,3,8,9] Common to all the adhesive ligands of the GPIIb/IIIa molecule is the three amino acid sequence arginine-glycine-aspartic acid (RGD). Peptides that contain this sequence

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Presented at the Forty-fifth Annual Meeting of the Society for Vascular Surgery, Research Forum I, Boston, Mass., June 4-5, 1991.

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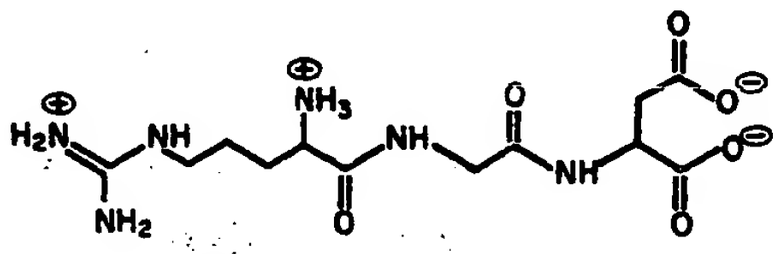
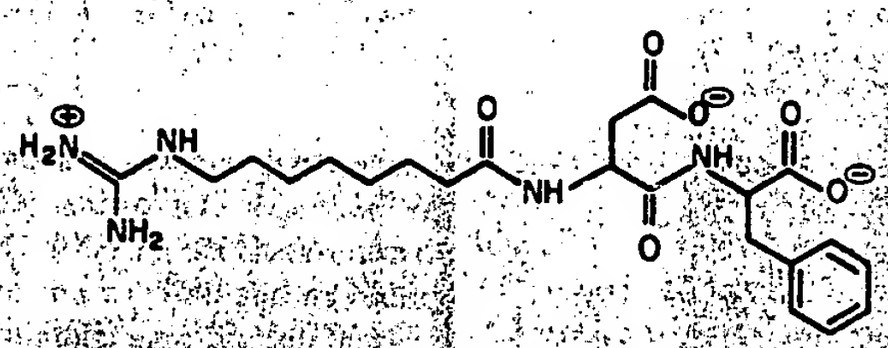


Arg-Gly-Asp (RGD)



8-Guanidino-octanoyl-Asp-Phe (SC#49992)

Fig. 1. The chemical structure of the native RGD sequence and the RGD analogue.

competitively inhibit the binding of all ligands.[2,10-12] In addition, some ligands have additional binding sites; for example, there is a non-RGD containing dodecapeptide sequence of fibrinogen that also binds GPIIb/IIIa.[13,14]

Earlier studies in our laboratory showed that peptides containing the arg-gly-asp sequence competitively inhibit the binding of the relevant ligands of the GPIIb/IIIa complex and thus effectively inhibit platelet aggregation in vitro.[11] We have previously used a canine model of early vascular graft occlusion to demonstrate that RGD containing peptides could significantly decrease acute platelet deposition in small diameter vascular grafts. These studies, however, were hampered by the extremely short half-life of the RGD peptide, necessitating intraarterial infusion immediately proximal to the graft.[15] More recently, 8-guanidino-octanoyl-Asp-Phe, a molecule that mimics the functional characteristics of the RGD sequence, has been synthesized (Fig. 1).

This study consists of two complementary parts. Using human platelets, in vitro experiments were performed to evaluate the effectiveness of SC-19992 in decreasing platelet aggregation, secretion, and adhesion. The second part of the study examined the ability of the RGD analogue to decrease platelet aggregation and to prevent platelet deposition on vascular grafts in the thrombosis prone canine model.

MATERIAL AND METHODS

Human platelet studies

In vitro assays. Fibronectin was purified by human plasma from affinity chromatography on gelatin sepharose as described.[4] Fibrinogen (grade L) was obtained (Kabi, Stockholm, Sweden). Bovine thrombin and prostaglandin E_1 (PGE_1) was purchased (Sigma Chemical Co., St. Louis, Mo.). Radiochemicals were from New England Nuclear (Boston, Mass.) (^{14}C serotonin) and ICN, Inc. (Irvine, Calif.) ($Na^{51}CrO_4$). Sepharose was derivatized with the RGD containing peptide VVTG-STRGDQSSWK, which was synthesized by standard solid phase methodology. Ninety-six well microliter plates were purchased from USA Scientific (Ocala, Fla.).

Platelet preparation. For adhesion experiments, human platelets from many healthy donors who had not been exposed to platelet active medications were isolated from freshly drawn whole blood anticoagulated with 1/10 volume acid-citrate-dextrose as previously described under protocols approved by the Human Studies Committee.[11] For platelet aggregation and secretion studies, platelets were prepared from freshly drawn whole blood in 3.8% sodium citrate, pH 7.4, with preparation of platelet-rich plasma (PRP) by the technique previously described.[11]

Adhesion studies. After preparation of PRP, 1 mm^3/ml of PGE_1 in a solution of tris-buffered saline (TBS) (0.05 mol/L tris-HCl, 0.15 mol/L NaCl, pH 7.4) was added and the platelets were washed once and resuspended in 1 ml of TBS and PGE_1 for 30 minutes with 50 mm^3 of ^{51}Cr. The labeled platelets were washed twice with TBS-PGE_1 and 1/10 volume acid-citrate-dextrose, and resuspended in TBS, bovine serum albumin, and glucose (TBG) (TBS plus 0.09% wt/wt D-glucose and 0.5% wt/wt bovine serum albumin) to obtain a platelet

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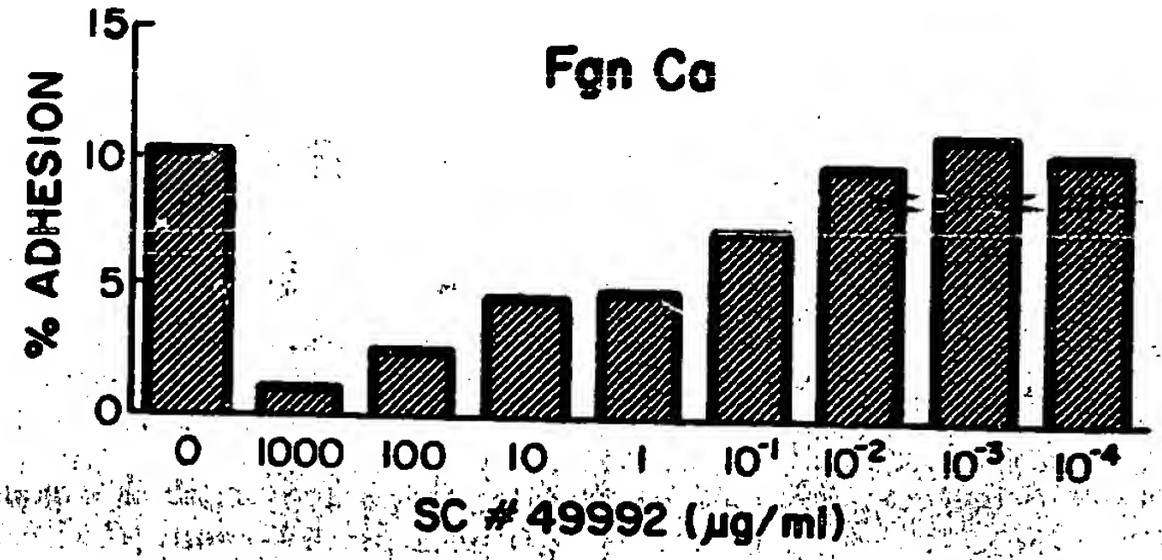

Fig. 2. Adhesion of thrombin stimulated human platelets in the presence of calcium to a fibrinogen substrate *(far left bar)*. The ability of the RGD analogue to inhibit thrombin stimulated platelet adhesion is demonstrated at concentrations $> 10^{-2}$ µg/ml.

count of 100 to 150,000 platelets/mm³. Either $CaCl_2$ or ethylenediamine tetraacetic acid (EDTA) was added to obtain a final concentration of 2 mmol/L in the TBG. A standardized method of platelet adhesion assays have been previously described by our laboratory with slight modifications for the purposes of these experiments.[11] SC-49992 and/or thrombin (final concentration 1 U/ml) were added as 1% vol/vol in a test tube and after a 5-minute incubation period without shaking, the solutions were pipetted onto precoated microtiter 96-well plates. These plates had been precoated with 100 mm³ of TBG, 25 µg/ml of fibrinogen in TBS, or 20 µg/ml of fibronectin in TBS for 2 hours. Platelet adhesion to bovine serum albumin in the presence or absence of calcium, to fibrinogen in the presence or absence of calcium, and to fibrinogen and calcium with added SC-49992 was measured. As well, dose response relationships over the range from 1 mg/ml of the RGD analogue to 0.1 ng/ml of the analogue were measured by use of quiescent and thrombin stimulated platelets. Identical experimental conditions were performed on fibronectin substrates. Aliquots of the solution containing the RGD analogue, thrombin, and labeled platelet mixture were counted in a gamma counter to determine their specific activity, and in a Coulter counter for determination of platelet count. After 1 hour of adhesion at 25° C, the microtiter wells were washed five times with 150 mm³ of TBG-Ca or TBG-EDTA buffer solutions. The adherent ^{51}Cr labeled platelets were solubilized with 2% sodium dodecyl sulfate, and the counts measured in a gamma counter.

Aggregation and secretion studies. Aggregation and secretion studies were carried out concurrently. Studies using adenosine diphosphate (ADP) as a stimulus were performed in PRP. Experiments performed with thrombin stimulated platelets used washed platelets in either TBS or Hepes-Tyrodes buffers with 2 mmol/L $CaCl_2$ or EDTA. Platelet-rich plasma was labeled with 30 mm³ ^{14}C serotonin for each 10 ml of PRP and incubated for 30 minutes at room temperature. Aggregation was measured in a Payton dual channel aggregometer (Buffalo, N.Y.). The reaction consisted of 400 mm³ of the platelet suspension, 50 mm³ SC-49992 or the appropriate control vehicle and, after a 5-minute preincubation period, 50 mm³ of ADP or thrombin. Aggregation studies were performed for 5 minutes after the addition of thrombin or ADP. The aggregometry sample was then rapidly transferred to a Eppendorf microcentrifuge and spun for 2 minutes at 11,000 *g* with determination of platelet ^{14}C serotonin secretion as previously described.[16] Final agonist concentrations for platelet stimulation were 10 µmol/L ADP and thrombin 0.2 U/ml.

Column chromatography experiments. The GP IIb/IIIa complex was purified from the particulate fraction of platelets produced by two cycles of freezing and thawing. Particulate material was collected by centrifugation and solubilized in extraction buffer (0.05 mol/L tris-HCl, 0.15 mol/L NaCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, a 50 mmol/L octyl-glucoside, pH 7.4). Protease inhibitors (PMSF 2 mmol/L, aprotinin 2 mmol/L, and leupeptin 2 mmol/L) were included in the extraction buffer. After centrifugation at 20,000 *g* for 45 minutes (4° C), the soluble supernatant was applied to a concanavalin-sepharose column equilibrated in the above buffer. After extensive washing, glycoproteins bound to the column were eluted with column buffer containing 0.5 mol/L alphamethylmannoside. Eluted glycoproteins were then subjected to affinity chromatography over an RGD derivatized sepharose

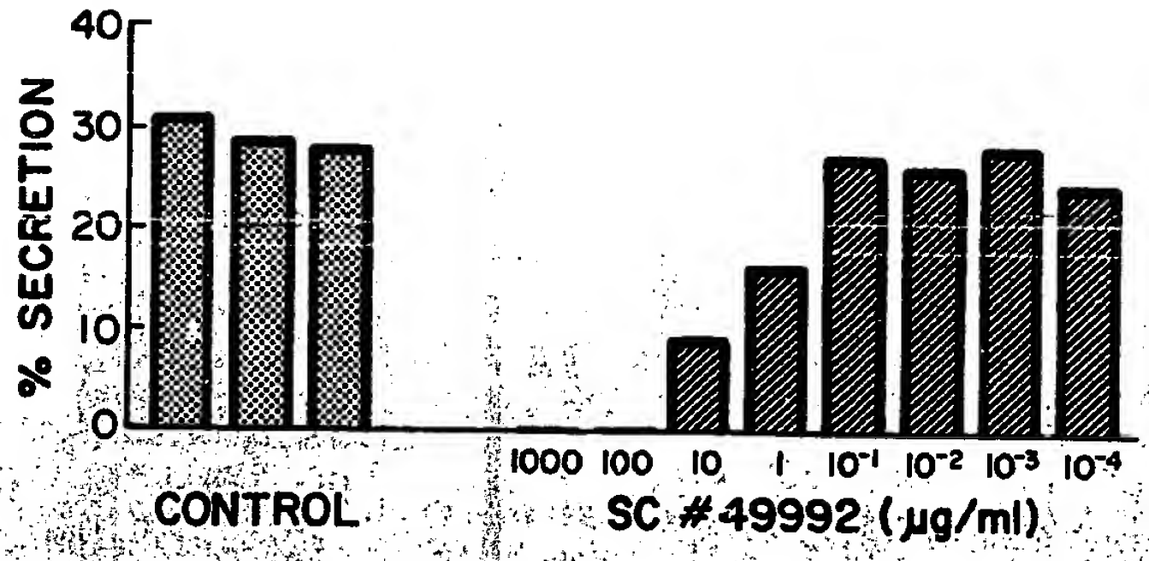

Fig. 3. ^{14}C serotonin secretion of ADP stimulated human platelets. Three control values depict percent secretion at beginning, middle, and end of experimental time period. SC-49992 was able to inhibit ADP induced ^{14}C serotonin secretion from human platelets when RGD analogue was present in concentrations >0.1 µg/ml.

column. Selected fractions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, with silver staining.

Canine vascular graft studies

Graft materials. Expanded polytetrafluoroethylene (PTFE; Gore-Tex, W. L. Gore and Assoc., Elkton, Md.) grafts measuring 4 mm × 14 cm were used.

Dogs. Eight mongrel dogs whose platelets exhibited irreversible arachidonic acid induced platelet aggregation were selected for implantation of the PTFE grafts. All animals were treated in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1985). Protocols were approved by the Washington University Committee on the Humane Care of Laboratory Animals.

8-guanidino-octanoyl-Asp-Phe. 8-guanidino-octanoyl-Asp-Phe was a generous gift of Dr. Steven P. Adams of the Monsanto Company, St. Louis, Mo., and was dissolved in a normal saline solution and buffered to a pH of 7.4.

Indium labeling. Before surgery 50 ml of blood was drawn from each animal and isolation of PRP was carried out as previously described.[11] Platelets were then labeled with indium III as previously described.[1]

Surgical technique. The eight thrombosis prone mongrel dogs weighing 20 to 30 kg were used. Anesthesia was obtained with intravenous administration of pentobarbital in an initial dose of 30 mg/kg. Smaller doses were then given at intervals as needed. After the induction of anesthesia, each animal was intubated, placed on a respirator, and given intravenous lactated Ringer's solution at 15 ml/kg/hour. The femoral artery was exposed and mobilized over a 10 cm distance. The dog was then systemically heparinized (100 U/kg) and atraumatic vascular clamps placed 4 cm apart. The femoral artery was divided between the clamps, and a 14 cm Gore-Tex graft with a 4 mm diameter was interposed in an end-to-end fashion by use of 7-0 Gore-Tex suture. A 1 mg/kg bolus of the RGD analogue was given, followed by a 50 µg/kg/min infusion of the agent. Alternatively, in the control condition, saline was used. Indium III–labeled platelets were reintroduced into the circulation. The clamps were released and blood flow restored through the graft and into the distal vasculature of the leg. Flow through the graft was measured throughout the experiment by means of a flow probe placed immediately proximal to the graft. Flow was maintained at 100 to 200 ml/min throughout the experimental period. Blood samples were drawn at time zero, that is, at the release of the clamps, and at 30-minute intervals until the conclusion of the experiment at 2 hours. Platelet count, radiolabeled platelet specific activity, and intraoperative ex vivo aggregometry were performed. Care was taken throughout the procedure to minimize radioactive contamination of the outside of the graft as a result of suture hole bleeding. The infusion of drug or control saline was stopped at 2 hours, and a ligature was placed proximally and distally to the anastomoses, and the graft was explanted. The graft was carefully irrigated with 50 ml of saline, placed in formalin, and counted in a gamma counter. Later, histologic examination of each graft was performed as described below. The following week in each dog the contralateral femoral position was used for graft placement. Dogs were randomly assigned to receive either the drug or saline the first week and the other substance 1 week later.

Analysis. Whole blood drawn at 30-minute intervals during the course of the experiment was separated into platelet-free plasma and PRP. The platelet count of the PRP was determined as well as the number of counts per minute (cpm) contained therein as measured by a Searle model 1197 (G. D. Searle and Co., Chicago, Ill.) automated gamma counter. This allowed calculation of the number of counts per minute per platelet (platelet specific activity). Activities at time 0, 30, 60, 90, and 120 minutes were then averaged. Results were calculated to determine the number of platelets deposited per square centimeter of graft by the formula: platelets/cm² = total graft counts/average platelet specific activity times graft surface area. A value of 17.9 cm² was calculated to represent the internal surface area of a 4 mm × 14 cm graft. To determine the efficacy of the labeling procedure, before reinfusion of the labeled platelets, the number of counts contained both in the PRP and platelet-poor plasma fractions were calculated to determine the amount of free and platelet bound indium. In all experiments, greater than 95% of the indium was platelet bound.

Histology. After determination of counts per minute, the grafts were submitted for histologic examination for both routine hematoxylin and eosin and Carstairs staining.

RESULTS

Human platelet studies

Platelet adhesion studies. Activation dependent platelet adhesion to fibrinogen substrates was reduced in a concentration dependent manner from 10.3% ± 0.7% to 1.0% ± 0.4% by SC-49992, with an ED_{50} of 1 μg/ml (Fig. 2). No significant inhibitory effect was noted with doses below 10 ng/ml. Maximal inhibition was seen at a concentration of 1 mg/ml of the analogue and reduced stimulated platelet adhesion by greater than 90%. Platelet adhesion to fibronectin substrates was also reduced from 10.1% ± 0.5% to 2.9% ± 0.6% by the analogue in a concentration dependent manner with an ED_{50} of 1 μg/ml.

Aggregation and secretion studies. Adenosine diphosphate induced aggregation and secretion was performed in PRP. Adenosine diphosphate induced aggregation and secretion is dependent on GPIIb/IIIa receptor occupancy by fibrinogen.[17] The ability of SC-49992 to inhibit both aggregation and secretion was evaluated as the inhibitor concentration was changed from 1 mg/ml to 0.1 ng/ml. RGD analogue concentrations greater than 1 μg/ml resulted in complete inhibition of aggregation. Half maximal aggregation was seen at concentrations in the range of 1 μg/ml, with restoration of full aggregation tracings by reducing inhibitor concentrations to 10 ng/ml or less (not shown). Adenosine diphosphate induced ^{14}C serotonin secretion was reduced to undetectable levels by the analogue in doses of 1 and 0.1 mg/ml. Restitution of control levels of secretion was seen with analogue concentrations of 0.1 μg/ml or less (Fig. 3).

Thrombin induced aggregation also depends on the ability of the platelet surface to bind fibrinogen. Thrombin induced aggregation was initially inhibited by the analogue, in the same concentration dependent manner seen with ADP induction. However, thrombin induced ^{14}C serotonin platelet secretion is not dependent on GPIIb/IIIa fibrinogen receptor binding. Thrombin induced ^{14}C serotonin secretion, in contrast to aggregation, therefore showed no inhibitory effect of the RGD analogue. This result is consistent with the observation of others that thrombin induced platelet activation and aggregation does not require the presence of exogenous fibrinogen.[18-20]

Elution experiments. Fig. 4 demonstrates the ability of 8-guanidino-octanoyl-Asp-Phe to effectively elute the GPIIb/IIIa complex from a column of RGD derivatized sepharose, as detected by silver staining. No additional proteins were detectable after elution by an EDTA containing solution.

Fig. 4. Silver stain of starting human platelet membrane preparation *(lane A)*. After passage over an RGD derivatized sepharose column, SC-49992 eluted proteins with characteristic electrophoretic mobility of subunits of GPIIb/IIIa in both reduced *(lane B)* and nonreduced *(lane C)* conditions.

Fig. 5. Transverse sections of midportion of PTFE graft after explantation (original magnification ×2.5). Hematoxylin and eosin staining of the graft demonstrates significant thrombus in control treated animals *(top left)*. Carstairs staining *(top right)* of an adjacent section of graft from same control treated animal demonstrates that this material is made up predominantly of platelets *(blue)* with some trapped red blood cells *(orange-brown)*. All sections, regardless of staining technique, from animals receiving RGD analogue infusion demonstrated essentially no identifiable thrombus *(bottom)*.

Table I. Platelet deposition on vascular grafts

Dog no.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Control (platelets × 10^6/cm²)	194	286	186	3100	191	119	317	147
Drug (platelets × 10^6/cm²)	2	7	68	11	6	27	26	34
% Reduction	99	98	63	99	97	77	92	98

Canine vascular graft experiments

Platelet deposition studies. The above in vitro experiments demonstrate that SC-49992 inhibits platelet aggregation by inhibiting the binding of platelet adhesive proteins to the GPIIb/IIIa complex. These results suggest that SC-49992 and related compounds may have clinical utility as antithrombotic agents. To test this concept we examined the ability of SC-49992 to inhibit platelet deposition on prosthetic vascular grafts. Table I shows the platelet deposition per square centimeter of graft under control and drug conditions for each dog. The RGD analogue reduced early platelet deposition from 63% to 99% (mean, 90%; $p = 0.0006$, log transform data, paired t test). The calculated reduction in platelet deposition was confirmed by the striking decrease in platelet deposition seen histologically (Fig. 5). It is noteworthy that in animal 3, which appeared to display the least effect of the drug, there was weeping of platelets through the interstices of the PTFE, as seen in Carstairs stained histologic sections. This allowed most of the platelets to be deposited on the exterior and within the interstices of the graft, and elevated the total platelet deposition.

Intraoperative aggregometry. Fig. 6 *(left)* shows the aggregation curves in response to arachidonic acid in one of the thrombosis prone dogs selected for this study. Fig. 6 *(middle)* shows the inability of platelets isolated during the drug infusion

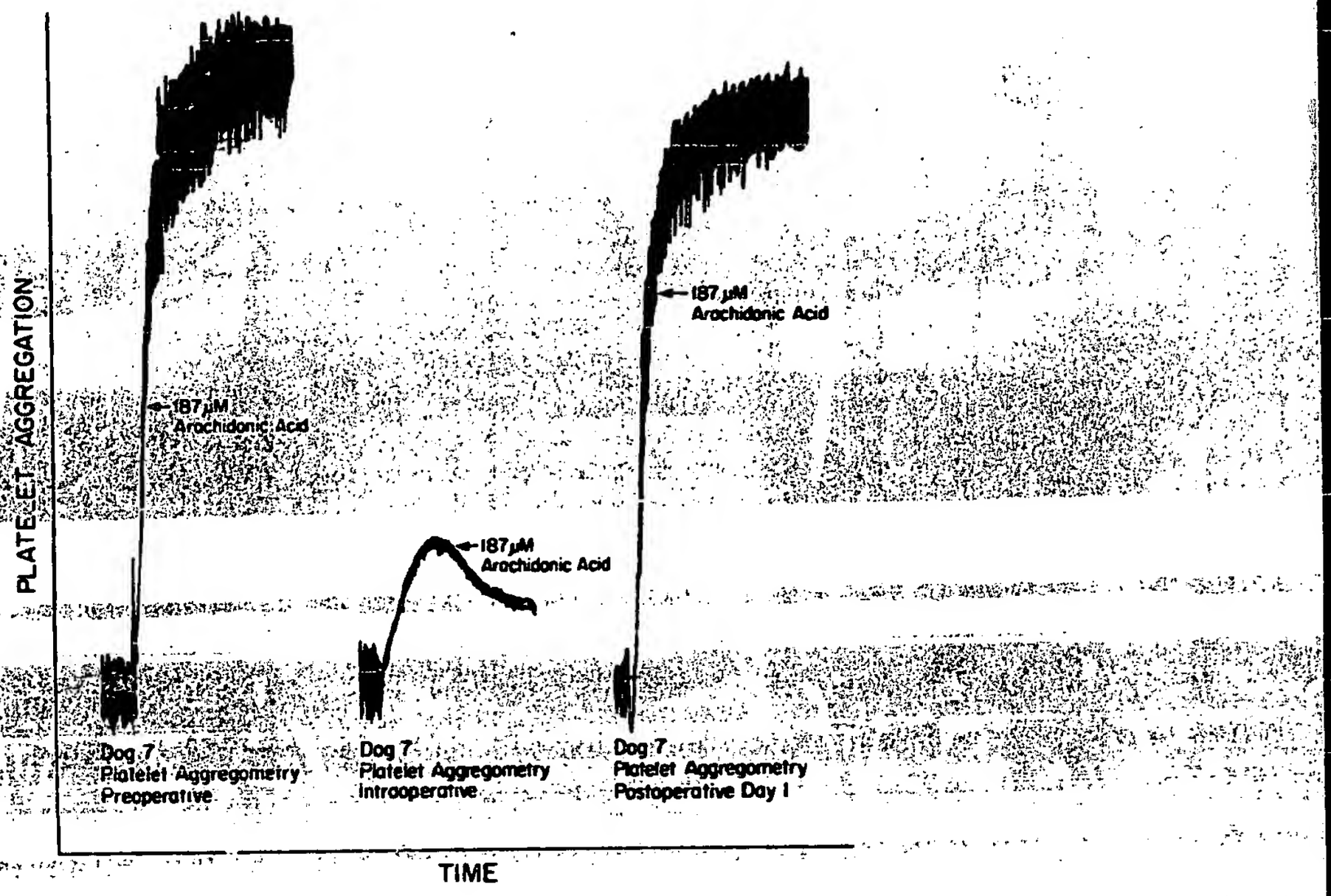

Fig. 6. Aggregometry tracings of dog no. 7. Preoperative aggregometry demonstrates prompt irreversible aggregation to arachidonic acid. Intraoperative aggregometry during infusion of RGD analogue demonstrates reduced and reversible aggregation to same agonist. Twelve hours after termination of the SC-49992 infusion, full arachidonic acid–induced aggregation is seen.

to aggregate. Fig. 6 *(right)* shows recovery of platelet function 12 hours later.

DISCUSSION

The ability of the RGD analogue to inhibit thrombin induced binding of human platelets to matrix proteins was significant at low concentrations, and appears to be at least as effective an inhibitor as the native RGD sequence. The ability of SC-49992 to inhibit ADP induced aggregation and secretion verifies effective inhibition of the GP IIb/IIIa complex, although the fibronectin and vitronectin receptors may be affected as well. Despite the presence of SC-49992, the platelets remain capable of full secretory response when stimulated with thrombin, which suggests that the inhibitory effect occurs at a postactivation stage, and is not a nonspecific toxic effect on the platelets. The RGD analogue was capable of initially inhibiting thrombin induced aggregation in the washed platelets, but as secretion occurred with release of endogenous platelet fibrinogen, a fibrin clot eventually formed in the aggregation vial. The requirement of GP IIb/IIIa receptor occupancy for effective ADP induced secretion is well documented, and the ability of the RGD analogue to inhibit ADP induced secretion again suggests potent inhibitory activity at the GP IIb/IIIa site. As well, elution experiments with column chromatography showed the ability of SC-49992 to remove all the previously bound GP IIb/IIIa from RGD-derivatized sepharose, with no other integrin eluted after stripping the column with EDTA. These results are consistent with the concept that the inhibitory effect of SC-49992 is due to its ability to inhibit binding of fibrinogen to the GPIIb/IIIa complex.

The results of the in vivo portion of the current study demonstrate the ability of the RGD analogue 8-guanidino-octanoyl-Asp-Phe to impair platelet aggregation and reduce platelet deposition in small diameter vascular grafts in a thrombosis-prone canine model. These findings are consistent with the previous work from our laboratory showing the efficacy of the RGD peptide as an antiplatelet agent in a similar model.[15] In contrast to other antiplatelet agents, this RGD analogue offers several distinct advantages.

Because GPIIb/IIIa represents the final common

pathway of platelet aggregation, the pharmacologic interdiction f the exposure and activation of this receptor has been carefully studied. Some agonists of platelet activation initiate the release of arachidonic acid through the cyclooxygenase pathway leading to the production of thromboxane A_2; the latter contributes to GPIIb/IIIa exposure and activation both through the release of platelet storage granules and directly through a receptor mechanism.[21] Aspirin, which inhibits cyclooxygenase, is the most commonly used agent and is effective at practical doses. Several agents inhibit thromboxane synthase yet require suprapharmacologic doses.[21] Because of this, other agents that block the receptor that mediates the effects of thromboxane A_2 and its cyclic adenosine monophosphate precursors on GPIIb/IIIa exposure have been developed.[21] The practical usefulness of all these agents is circumscribed by thromboxane A_2 not being absolutely necessary for GPIIb/IIIa exposure and their lack of platelet specificity.[21]

Other agents that modulate cyclic monophosphate nucleotides are theoretically attractive in that they inhibit all activation methods. However, these agents are neither platelet specific nor without side effects. For instance, endothelium-derived relaxing factor, thought to be nitric oxide, putatively modulates platelet cyclic guanosine monophosphate.[23] Other novel agents such as ticlopidine inhibit GPIIb/IIIa exposure by incompletely elucidated mechanisms and are not without side effects.[23]

Because of the theoretic and practical limitations of blocking GPIIb/IIIa exposure and activation, another pharmacologic strategy has been to block the already exposed GPIIb/IIIa receptor itself. Monoclonal antibodies interacting with either the alpha of beta subunit of GPIIb/IIIa or the entire complex have been shown to inhibit fibrinogen binding, as well as that of other adhesive proteins, and to produce a thrombasthenic like state in normal platelets in vitro.[24] Hanson et al.[25] were able to demonstrate the efficacy of monoclonal antibodies to GPIIb/IIIa in reducing platelet aggregation and deposition on Dacron grafts, but they were unable to show a reduction in thrombotic graft occlusion. Coller et al.[26] demonstrated the ability of anti-GPIIb/IIIa antibodies to normalize blood flow in a model of platelet dependent coronary artery stenosis. As well, anti-GPIIb/IIIa antibodies have been shown to augment the effects of tissue-plasminogen activator.[27] Despite the theoretic advantages of using anti-GPIIb/IIIa antibodies as platelet inhibitors, this approach is limited by availability, cost, irreversibility, and most importantly, by antigenicity, which limits the number of applications in any one patient.

A significant advantage of SC-49992 lies in the rapid resolution of its platelet inhibitory effects once infusion is terminated. With a pharmacologic half-life of approximately 10 minutes,[28] and full recovery of collagen induced platelet aggregation within 30 minutes,[29] rapid return of normal platelet function allows for resolution of any bleeding problems associated with drug infusion. Further characterization of the pharmacologic properties of SC-49992 have recently been described in abstract form.[30,31]

Bleeding complications. Concern exists about the bleeding diathesis associated with RGD sequence inhibition. In the canine graft platelet deposition studies, the only sites of persistent hemorrhage were the needle holes in the graft. Despite what was likely a suprapharmacologic dose of SC-49992 in addition to systemic heparinization, the remainder of the operative field appeared hemostatic.

The concept of RGD inhibition may not be without inherent problems. The RGD peptide serves as a recognition sequence for adhesive receptors such as the fibronectin and vitronectin receptor. These are used by other cell types including vascular endothelial cells to adhere to components of the extracellular matrix.

The RGD analogue 8-guanidino-octanoyl-Asp-Phe extends the concept of RGD inhibition of GPIIb/IIIa to a clinically useful form. Intravenously administered and rapidly reversible, it is a potent inhibitor of platelet aggregation and deposition. Potential uses include the treatment of transient ischemic attacks, concomitant administration with tissue plasminogen activator to potentiate thrombolysis and reduce platelet mediated recurrent thrombosis,[28] and applications where antiplatelet agents such as dextran are currently used. Further studies will be needed to determine this agent's therapeutic window, adverse side effects, and to better define its role in the clinical armamentarium.

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DISCUSSION

Dr. William C Krupski (San Francisco, Calif.). I congratulate the authors for performing an interesting, well-conducted study and for presenting it today so clearly. Their findings are extremely important in our evolving armementarium for preventing thrombosis. The initial events responsible for the attachment of platelets to thrombogenic surfaces are determined by complex interactions of subendothelial components and well-characterized receptors on platelet membranes.

One such receptor, the platelet glycoprotein IIb/IIIa complex, interacts with diverse adhesive proteins, the most important of which is fibrinogen. GP IIb/IIIa is a

calcium-dependent heterodimer belonging to a general class of structurally similar adhesion receptors called integrins, which interact with the amino acid sequence arginine-glycine-aspartic acid common to fibrinogen and other proteins that mediate cell adhesion. Occupancy of this receptor by fibrinogen is the primary pathway for platelet aggregation. Therefore, GPIIb/IIIa is an important target for interventions aimed at interupting clinically significant, pathologic thrombus formation.

Rubin et al. have investigated a new peptide containing the arg-gly-asp sequence that competitively binds to the GPIIb/IIIa receptor on platelets. They convincingly showed that infusion of this peptide resulted in a 90% decrease in platelet deposition on small caliber prosthetic grafts, a clinically relevant model of thrombosis.

To fully appreciate the importance of these observations, it would be helpful to consider some of the other approaches to antiplatelet therapy. Interruption of the arachidonic acid pathway is the most commonly used strategy. Aspirin, dipyridamole, nonsteroidal antiinflammatory drugs, thromboxane synthetase antagonists, steroids, and calcium blocking agents each work in a slightly different way, but all interfere with the vasoconstrictive and aggregatory effects of thromboxane. The critical reader of the literature may be confused by contradictory claims about these drugs, which confound interpretation of results.

Thrombin plays a central bioregulatory role in hemostasis and is a potent stimulus for platelet activation by a mechanism that is independent of ADP, thromboxane, and fibrin generation. Thus, inhibition of thrombin is an effective way to prevent undesired thrombosis. The list of antithrombins that have been evaluated continues to grow, and earlier this afternoon we heard about hirudin, a potent biologic antithrombin. However, because the new antithrombins so completely block thrombus formation, hemorrhagic complications are a problem.

Directly blocking the GPIIb/IIIa receptor on platelets is a particularly attractive antiplatelet strategy. Three categories of agents have been evaluated for this purpose. The first includes these natural RGD-containing proteins that have been isolated from the venom of several vipers, including echistatin, bitistatin, and applagin.

The Monsanto product described by Dr McGraw this afternoon is but one of several synthetic peptides with one or more RGD sequences currently under evaluation.

A third strategy to ablate platelet-thrombus formation uses monoclonal antibodies that specifically bind to and inhibit GPIIb/IIIa. We have studied monoclonal antibodies to this receptor in a model of thrombosis consisting of segments of aorta interposed in an external arteriovenous shunt in baboons. Platelet deposition on the endarterectomized arteries was pronounced. Treatment of animals with the GPIIb/IIIa antibody, CP-8, virtually abolishes platelet deposition on this thrombogenic surface, reducing thrombus formation to the level of control, unendarterectomized arterial segments.

I have several questions for Dr McGraw that relate to potential clinical relevance of his findings. First, was there a drop in platelet count during infusion as has been reported with other receptor antagonists? Second, what is the reason for the dosage regimen chosen? Were drug levels measured and constant? Do you know the dose response? Third, is there any lasting effect of this drug, or will platelets reaccumulate as soon as infusion stops? Were any cardiopulmonary or other complications encountered? Can the authors put this agent in perspective with some of the other strategies I discussed, and do they have any information about the status of compounds being developed by other companies?

Dr. Daniel J. McGraw. I thank Dr. Krupski for his comments and questions. I will try to answer his questions in order. During infusion of the RGD-analogue for the canine experiments, platelet counts were measured every 30 minutes. Throughout the period of infusion there were no alterations in platelet counts, nor did the platelet counts during infusion differ from those before infusion. Second, Dr. Krupski asked how we selected the dosage regimen. Our in-vitro studies on inhibition of platelet adhesion, secretion, and aggregation suggested the concentration necessary to achieve full inhibitory efficacy. We did not measure serum levels of the drug during infusion, but rather chose to use a dosage regimen calculated to be at the high end of the inhibitory spectrum. Despite the use of a high dose of the RGD-analogue, we saw full restoration of arachidonic acid–induced aggregation 12 hours after analogue infusion was terminated. Other groups have shown a return of platelet aggregatory activity as soon as 30 minutes after the end of infusion. We did not witness any cardiopulmonary disorders after drug infusion in the canine experiments. As expected, performing a prosthetic arterial bypass in an animal who is systemically heparinized and is receiving potent platelet inhibition resulted in some hemorrhagic problems. Surprisingly, these were relatively mild and consisted only of needle hole bleeding from the prosthetic side of the arterial suture lines. These were relatively easy to control, and we made a special effort to keep any extravasated blood from contaminating the outside of the prosthetic grafts. After the infusion was terminated and the grafts were removed, we did not encounter any ongoing problems during wound closure, nor after operation with wound hematomas. In response to Dr. Krupski's final question, we do not have any information about the status of compounds being developed by other companies. Our purpose, however, is not to champion this specific RGD-analogue, but rather to advance the concept of the potential clinical utility of RGD inhibition of the GPIIb/IIIa complex.

STIC-ILL

From: Gabel, Gailene
Sent: Monday, January 06, 2003 5:03 PM
To: STIC-ILL
Subject: 09/853,524

Please provide a copy of the following literature ASAP:

1) Rubin et al., New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. Journal of Vascular Surgery, (1992) 15/4 (683-692).

2) Armstrong et al., Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection), THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.

3) ELY et al., PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA, CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904.

4) Bastida et al., Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin, and phospholipase. Cancer Research, (1982) 42/11 (4348-4352).

5) Lee et al., In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency. BLOOD, (1979 Mar) 53 (3) 465-71.

6) Cattaneo et al., Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. BLOOD, (1990 Mar 1) 75 (5) 1081-6.

7) Wang et al., Exogenous adenosine application inhibits thrombus formation in stenosed canine coronary artery and partially protects against renewal of thrombus formation by epinephrine. FASEB Journal, (1995) Vol. 9, No. 3, pp. A322.

Thanks a bunch,
Gailene R. Gabel
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Pergamon

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INHIBITION OF RED BLOOD CELL-INDUCED PLATELET AGGREGATION IN WHOLE BLOOD BY A NONIONIC SURFACTANT, POLOXAMER 188 (RHEOTHRX® INJECTION)

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Abstract RheothRx Injection, an aqueous solution of a nonionic block copolymer (poloxamer 188) formulated for intravenous administration, was investigated as an inhibitor of red blood cell (RBC)-induced platelet aggregation at plasma concentrations of 0.05-5mgmL^{-1}. Platelet aggregation was determined by measuring the fall in single platelet counts after mechanical agitation of 2mL aliquots of citrated whole blood in a 37°C shaking waterbath. Inhibition of RBC-induced platelet aggregation of >95% was observed for poloxamer 188 at a concentration of 1mgmL^{-1}, and 41% inhibition was observed at 0.05mgmL^{-1}. Poloxamer 188 was observed to be a more effective inhibitor of RBC-induced platelet aggregation than 2-chloradenosine (2-ClAd) or phosphoenolpyruvate/pyruvate kinase (PEP/PK). Studies using platelet rich plasma (PRP) showed that platelet aggregation could not be induced by shaking in the absence of RBC, though aggregation was induced by the addition of exogenous adenosine diphosphate (ADP). Poloxamer 188 did not inhibit ADP-induced platelet aggregation. We propose that poloxamer 188 protects RBC from mechanical trauma by non-specific adsorption of copolymer to the RBC surface (via the hydrophobic polyoxypropylene moiety), and that this effect prevents mechanical damage and hence leakage of ADP from RBC. RheothRx Injection has been shown to have value in the treatment of acute ischemic disorders such as myocardial infarction. The observation of significant inhibition of RBC-induced platelet aggregation at clinically relevant concentrations suggests that RheothRx Injection may have antithrombotic properties *in vivo*, and may therefore have potential not only in acute ischemia but also to prevent thrombosis within vascular prostheses or to prevent rethrombosis after angioplasty or endarterectomy.

Key words: platelet aggregation, erythrocytes, RheothRx Injection, poloxamer 188, Pluronic F68.

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Poloxamers are nonionic ABA bl ck copolymers f polyoxyethylene (A) and p lyoxypropylene (B) (Fig. 1) and are commercially available over a wide range of molecular weights (1000 to 14000 $gmol^{-1}$) and polyoxyethylene/polyoxypropylene rati s. These copolymers are produced commercially under several tradenames [e.g. Pluronics (BASF, Wyandotte, MI.), Synperonic PE nonionic surfactants (ICI, Middlesbrough, U.K.)] and have numerous industrial uses (1-3) for example as emulsion stabilizers, foaming, defoaming, antistatic and wetting agents. The desired hydrophobic/hydrophilic balance is achieved by varying the total molecular weight and polyoxypropylene/polyoxyethylene ratio.

$$HO—(CH_2CH_2O)_A—(CH(Me)CH_2O)_B—(CH_2CH_2O)_A—H$$

FIG. 1.

Generalized structure of a poloxamer, where A is polyoxyethylene and B is polyoxypropylene.

Research into possible applications and therapeutic uses of poloxamers has been focused on poloxamer 188 (total molecular weight = 8400$gmol^{-1}$ with polyoxypropylene = 1750$gmol^{-1}$, 80% polyoxyethylene), ranging from drug delivery systems (4), topical formulations (5), hemorheology (6) to plant growth stimulation (7). Poloxamer 188 has been shown to reduce sequestration of labelled polystyrene nanospheres by the liver and prolong their circulatory times (4). As an additive to the priming fluid for bubble oxygenators in cardiopulmonary bypass pumps, poloxamer 188 reduced platelet adhesiveness and blood viscosity (8,9). RheothRx® Injection is a 15% (w/v) aqueous solution of poloxamer 188 formulated for intravenous injection, developed by Burroughs Wellcome Co. (Research Triangle Park, NC). RheothRx Injection has been shown to improve blood flow in ischemic tissue (10,11) and to have an antithrombotic effect *in vivo* (12), and is therefore potentially useful for the treatment of a variety of diseases characterized by acute vaso-occlusion or impaired blood flow [e.g., myocardial infarction, sickle cell disease (13)].

Hemorheologic effects of poloxamer 188, e.g., decreased blood viscosity and reduced RBC aggregation, have been clearly demonstrated *in vitro* at clinically relevant concentrations (6,9), and these effects are consistent with the observed *in vivo* effects on blood flow (10). In contrast, significant anti-platelet effects have only been detected *in vitro* at much higher concentrations than can be clinically achieved (14), a finding which is inconsistent with the apparent *in vivo* antithrombotic properties of RheothRx Injection. However, most previous *in vitro* platelet studies have examined the effect of poloxamer 188 on aggregation induced by the addition of exogenous agonists to platelet rich plasma (PRP) (14,15). Such studies thus neglect the role of RBC as a modifier of platelet function, as it has been shown that various platelet aggregation inhibitors and activators function very differently in whole blood compared to PRP (16-23). Further, RBC have been shown to induce platelet aggregation under conditions of shear (19), and this effect is thought to be a significant cause of platelet activation *in vivo* in situations characterized by localized regions of high shear stress such as caused by prosthetic heart valves, vascular grafts, or

regions in which the endothelium is damaged or diseased (20,24,25). Certain clinically useful antithrombotic agents, such as dipyridamole, which is effective in preventing platelet activation *in vivo*, do not inhibit platelet aggregation in PRP, but have been shown to significantly inhibit RBC-induced platelet aggregation in whole blood (20).

To address issues germane to RBC effects, Saniabadi et al. (18) developed a gentle shear technique to induce platelet aggregation by rollermixing citrated whole blood samples. The degree of platelet aggregation was determined by the fall in single platelet count, measured using a blood cell counter. This technique has been used to investigate the role of RBC in spontaneous or RBC-induced platelet aggregation, and also to determine the effects of platelet aggregation inhibitors [e.g., 2-chloradenosine (19), dipyridamole (20)], ADP-depleting enzyme/substrate systems [e.g., phosphoenolpyruvate / pyruvate kinase (PEP/PK) (26)] and the effects of inducers of platelet aggregation (e.g., ADP).

The technique described by Saniabadi et al. has been adapted here to study the effects of RheothRx® Injection (poloxamer 188) on RBC-induced platelet aggregation using a shaking waterbath at 37°C. The effects of RheothRx Injection were compared with various known inhibitors of platelet aggregation (2-chloradenosine and PEP/PK) and examined in the presence of the aggregating agent ADP. The results suggest that RheothRx Injection inhibits platelet aggregation at much lower concentrations than previously reported using PRP (14), and that this effect is primarily due to a cytoprotective action on RBC.

MATERIALS AND METHODS

Chemicals.
Poloxamer 188 was supplied by Burroughs Wellcome Co. as a 15% (w/v) sterile solution (RheothRx Injection), and was diluted with endotoxin-free isotonic phosphate buffered saline (Dulbecco's PBS, Sigma Chemical Co., St.Louis, MO), to give stock solutions of 25.00, 12.50 and 1.25mgmL^{-1}. Phosphoenol pyruvate (PEP), pyruvate kinase (PK), 2-chloradenosine (2-ClAd), and adenosine-5'-diphosphate (ADP) were purchased from Sigma and used as received without further purification. All stock solutions were prepared using Dulbecco's PBS.

Sample agitation.
Platelet aggregation was investigated using two methods of shaking. For poloxamer 188 concentration effects, an orbital shaking waterbath was used (Model 3545, Lab-Line Instruments Inc., Melrose Park, IL) at 37°C and 160 rpm, and for experiments comparing Poloxamer 188 with PEP/PK and 2-ClAd, a reciprocal shaking waterbath was used (Model 25, Precision Scientific Co., Chicago, IL) at 37°C and 90 oscillations per minute. Both methods gave qualitatively similar results, though the orbital shaking bath caused a somewhat greater degree of platelet aggregation.

Platelet count and hematocrit determinations.
Platelet counts were determined using an automatic cell counter (Minos STX, Roche Diagnostic Systems, Branchburg, NJ). Twenty microliter aliquots f samples of whole blood or platelet rich plasma were required to determine the platelet count at each time point. Hematocrits were measured using the microhematocrit technique (12,000g, 4 min).

Whole Blood and Platelet Rich Plasma Preparation.
Using a 19-gauge butterfly needle, blood was drawn slowly from the antecubital vein from healthy adults aged 25-40 years into four 10mL plastic syringes containing 1mL of citrate buffer as anticoagulant (32.0gL^{-1} sodium citrate, 4.2gL^{-1} citric acid, pH5.7, osmolality 332 $mOskg^{-1}$). No donors had taken medication known to affect platelet function (e.g. aspirin) for at least 72 hours prior to blood withdrawal. Following venipuncture, the blood was immediately transferred to a waterbath thermostatted at 37°C. Platelet Rich Plasma (PRP) was prepared by room temperature (22 ±1°C) centrifugation of anticoagulated blood at 150g x 15 min. Whole blood platelet counts were in the range 200-350x10^3mm^{-3} , while PRP platelet counts were in the range 400-550x10^3mm^{-3}.

Effect of RheothRx® Injection (poloxamer 188) on RBC-induced platelet aggregation.
Two milliliter aliquots of citrated blood at 37°C were transferred to stoppered cylindrical 50x16mm polystyrene flat-bottomed tubes (No.58.485, Sarstedt Inc., Newton, NC) containing a fixed volume (ca. 40µL) of appropriate stock poloxamer 188 solutions to give final plasma concentrations of 5.0, 1.0, 0.50 and 0.05$mgmL^{-1}$ poloxamer 188; volumes of stock poloxamer 188 solutions were based on whole blood hematocrit. The sample was mixed gently by inversion of the tube two times, and the platelet count was recorded at time 0. The sample was then placed horizontally in the orbital shaking waterbath and platelet counts recorded each minute for a total of 10 minutes. For each concentration of poloxamer 188, a control using an equal volume of PBS was studied, and each pair of experiments were performed in duplicate for each donor. The percentage inhibition of platelet aggregation was determined by comparing the decrease in the number of single platelets in the poloxamer 188-treated blood after 10 minutes to the decrease in the number of single platelets in the corresponding PBS control sample.

RheothRx Injection, PEP/PK, and 2-ClAd
Venous blood was collected as above but into 3mL plastic syringes containing 0.3mL of citrate buffer and 75µL of either PEP/PK (2.5mM PEP, 500UmL^{-1} PK), 2-ClAd (2.4mM), poloxamer 188 (25$mgmL^{-1}$) or PBS (control), yielding nominal plasma concentrations of 0.1mM/20UmL^{-1}, 0.1mM and 1.0$mgmL^{-1}$ for PEP/PK, 2-ClAd and poloxamer 188 respectively. For each additive, duplicate samples of blood were collected i.e., 8 syringes per donor. Two milliliters of each sample was transferred to stoppered cylindrical 50x16mm polypropylene flat-bottomed tubes (No.58.536, Sarstedt Inc., Newton, NC) and mixed gently by inverting the tube two times. The platelet count was recorded at time 0, the samples placed horizontally in the reciprocal shaking waterbath, and platelet counts were recorded after 15 and 30 minutes of agitation.

Platelet rich plasma and ADP-induced platelet aggregation.
Tw milliliters of PRP was transferred to a stoppered cylindrical 50x16mm polypropylene flat-bottomed tube containing 83μL of a 25mgmL^{-1} solution of poloxamer 188 (resulting copolymer concentration of 1.0mgmL^{-1}), and mixed gently by inverting the tube two times. The platelet count was recorded at time 0, the sample was then placed horizontally in the reciprocal shaking bath and the platelet count was recorded after 10 minutes of shaking. Forty three microliters of a 100μM solution of ADP in PBS was then added to the sample (resulting ADP concentration of 2μM). Following mixing by gentle inversion of the container, the platelet count was recorded 15 seconds post-ADP addition. The sample was then shaken in the waterbath and the platelet count recorded at 10 minutes post-ADP addition. A series of control experiments were also carried out using PBS in place of either the poloxamer 188 or ADP (83μL or 41μL of PBS respectively).

Statistical analysis of all data was by paired t-test.

RESULTS

Initial experiments comparing the effect of shaking on citrated whole blood and PRP were performed to evaluate the system and to address two specific issues: a) whether the observed platelet aggregation was due to mechanical shearing of the platelets; and b) whether the fall in single platelet count could be simply explained by adherence of platelets to the tube wall during shaking. Using whole blood without any additives, the total platelet count fell by approximately 50% after 10 min shaking at 160rpm. In contrast, it was found that the samples of PRP, when shaken in the absence of RBC (with or without poloxamer 188), showed no meaningful drop in platelet count over the time period investigated (up to 1 hour in total). However, after addition of an appropriate amount of packed RBC to the PRP samples at the end of the 1 hour shaking period in order to simulate whole blood, normal platelet aggregation was restored. These results indicate that: a) the presence of RBC is essential for platelet aggregation in this system; and b) there is minimal adherence of platelets to the surface of the tube. Note that there was also no difference in the degree of platelet aggregation observed between whole blood samples agitated in polystyrene or polypropylene containers, again indicating that the aggregation effects observed herein were not mediated by the surface of the containers.

RBC-induced platelet aggregation was significantly inhibited by poloxamer 188 as shown in Fig. 2 and in Table I ($p<0.001$). In the absence of poloxamer 188, the control platelet count fell by an average of 53% after 10 min shaking. In comparison, even at the lowest concentration of poloxamer 188 used for this study (0.05mgmL^{-1}), the platelet count fell by only 30% (41% inhibition, $p<0.001$ vs. control). At poloxamer 188 concentrations of 0.50, 1.0 and 5.0mgmL^{-1}, 82, 96 and 86% inhibition was observed respectively. Note that: a) the effects of poloxamer 188 plateau at the higher concentrations; b) for the 0.05, 0.50 and 1.0mgmL^{-1} data, the relation between poloxamer 188 concentration and percent inhibition is log-linear ($r = 0.99$, not shown); and c) fifty percent inhibition of platelet aggregation is achieved at a poloxamer 188 concentration of approximately 0.08mgmL^{-1}.

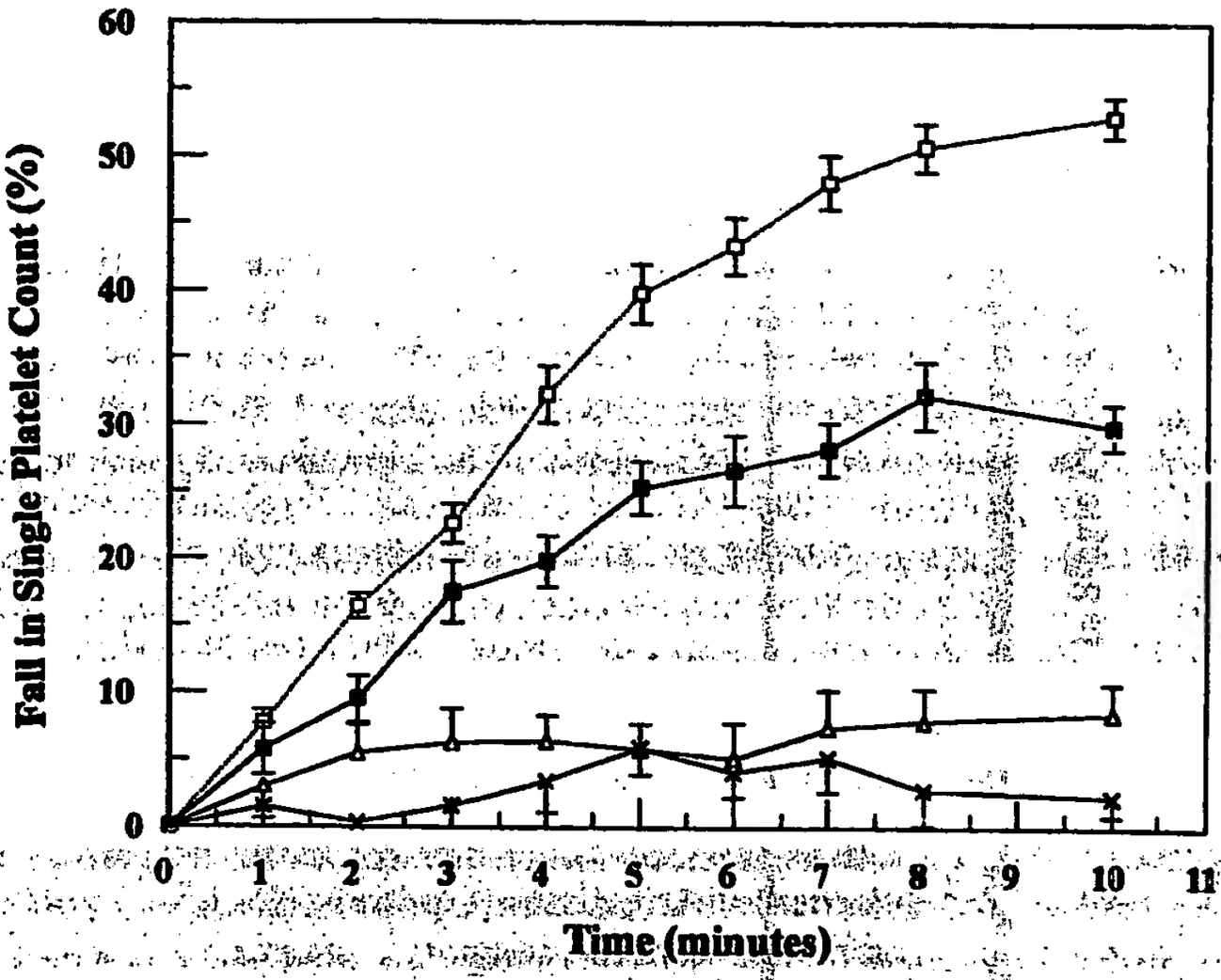

FIG. 2

Percentage fall in single platelet count for samples of citrated whole blood shaken at 90 oscillations per minute and at 37°C. Whole blood samples treated with (-·-□-·-) PBS control, (—■—) 0.05mgmL^{-1}, (—Δ—) 0.5mgmL^{-1} and (—✕—) 1.0mgmL^{-1} poloxamer 188 (mean ± sem, $p \leq 0.001$ versus control, n=5).

Addition of 2μM ADP to PRP resulted in an immediate drop in platelet count to approximately 25% of the original value in both poloxamer 188 and control PRP samples (Fig. 3). It is thus evident that poloxamer 188, at the dose studied (1.0mgmL^{-1}), has no effect whatever on the initial rate of ADP-induced platelet aggregation. Platelet counts recovered to approximately 80% of the original values for both control and 1.0mgmL^{-1} poloxamer 188 treated samples of PRP during continued shaking over the next 10 minutes. A similar percentage fall in platelet count was also observed after the addition of 2μM ADP to whole blood, either in the presence or absence of poloxamer 188 (data not shown). No fall in platelet count was observed for control experiments in which PBS was added to PRP or whole blood in place of ADP.

As shown in Table II, poloxamer 188 was observed to be a more potent inhibitor of RBC-induced platelet aggregation compared to the ADP-receptor blocker 2-chloradenosine or the ADP-depleting substrate-enzyme system PEP/PK ($p < 0.01$). In this series of experiments, an 80.4% inhibition of RBC-induced platelet aggregation was observed for poloxamer 188 at a plasma concentrati n of 1.0mgmL^{-1}, compared to a 37.1% inhibition for 2-ClAd at a plasma concentration of 100μM and a 55.0% inhibition for PEP/PK (0.1mM/20UmL^{-1}).

TABLE I

Effect of RheothRx® (poloxamer 188) Injection on RBC-induced platelet aggregation. Average single platelet counts for 2mL aliquots of citrated whole blood, shaken at 160rpm over 10 minutes at 37°C. (mean ± sem, n=5). *p<0.001 versus control.

Plasma Concentration of poloxamer 188 ($mgmL^{-1}$)	Single Platelet Count T=0 ($x10^3 mm^{-3}$)	Single Platelet Count T=10 minutes ($x10^3 mm^{-3}$)	Inhibition (%)
0.0	219 (±4)	102 (±3)	0.0
0.05	213 (±6)	149 (±5)	41.3* (±7.5)
0.50	230 (±10)	209 (±7)	82.2* (±4.6)
1.0	215 (±8)	210 (±8)	95.5* (±2.8)
5.0	219 (±10)	202 (±9)	85.9* (±5.6)

TABLE II

Comparison of RheothRx (poloxamer 188) Injection with 2-chloradenosine and phosphoenolpyruvate/ pyruvate kinase on RBC-induced platelet aggregation. Average percentage fall in single platelet counts of 2mL aliquots of citrated whole blood, shaken at 90rpm at 37°C over 30 minutes. (mean ± sem, n=5). *p<0.005 versus control.

	Fall in Single Platelet Count (%)			
Time of Agitation (minutes)	Control (PBS)	2-Chloradenosine (100µM)	Phosphoenolpyruvate/ Pyruvate Kinase (0.1mM/20UmL^{-1})	Poloxamer 188 (1.0$mgmL^{-1}$)
15	8.0 (±1.2)	8.1 (±1.1)	5.2 (±0.9)	2.9 (±1.5)
30	17.5 (±1.5)	10.4 (±1.3)	7.5 (±1.4)	3.2 (±1.8)
Inhibition at 30 min (%)	0.0	37.1* (±8.5)	55.0* (±9.4)	80.4 (±10.0)

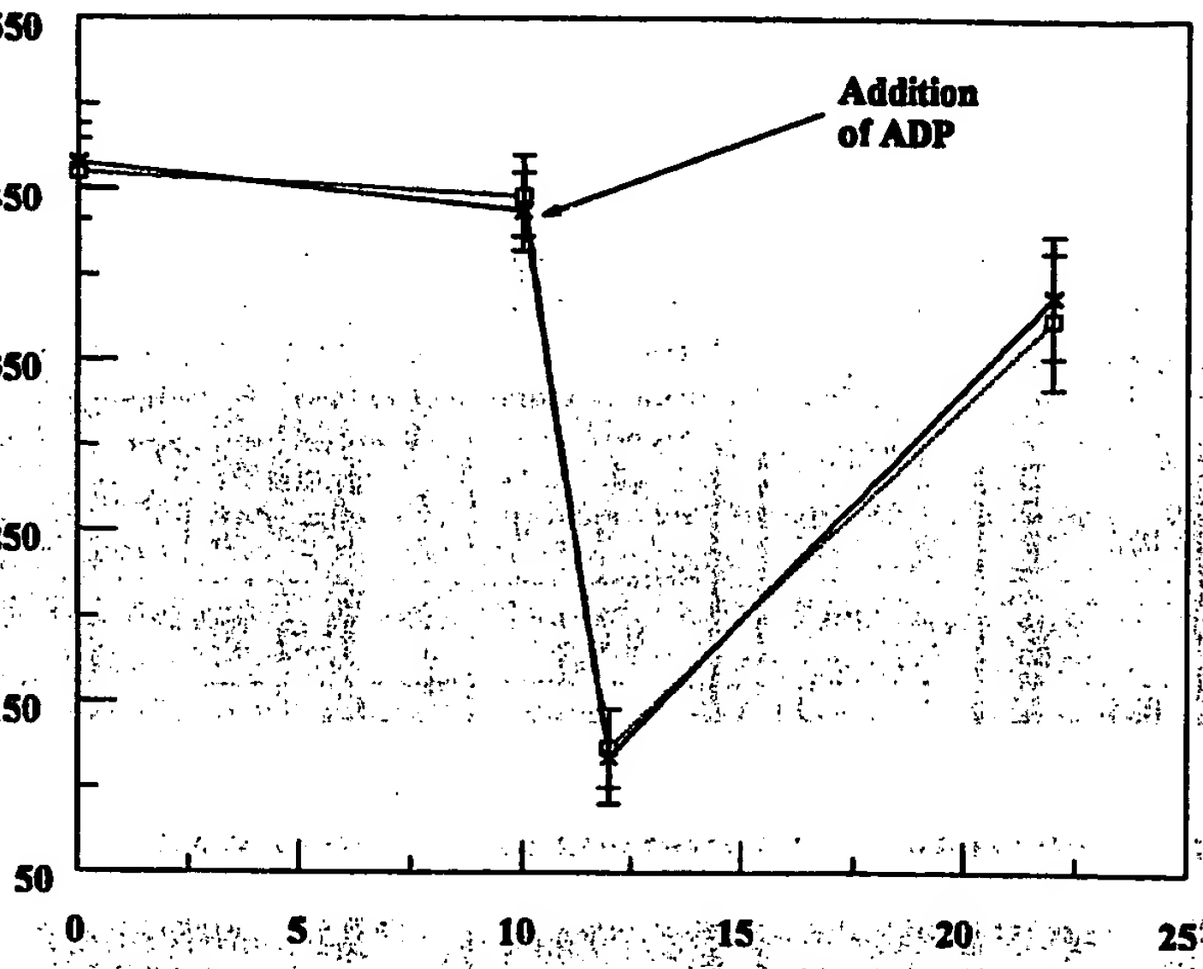

FIG. 3.

Fall in single platelet count for samples of PRP shaken at 90 oscillations per minute and at a temperature of 37°C treated with (—✕—) poloxamer 188 (1.0mgmL^{-1}) and (· ·□· ·) PBS (Control). Addition of 2μM ADP after 10 minutes shaking resulted in an initial 75% fall in the single platelet count, recovering to 20% of the original count for both poloxamer 188 and control samples after 10 minutes (mean ± s.d., n = 4).

DISCUSSION

RheothRx® Injection or other forms of poloxamer 188 have been evaluated in several animal studies and appear to be useful in a variety of clinical states characterized by acute vaso-occlusion or impairment of blood flow: Poloxamer 188 has been shown to reduce myocardial infarct size and improve left ventricular function after 90 minutes mechanical occlusion of the left anterior descending coronary artery in dogs (27,28), to reduce microvascular sequelae of burn injury in rats (29), to improve survival in dog and rabbit models of hemorrhagic shock (30-32), and to improve the patency rate of microvascular anastomoses in rabbits (33). Clinical studies in patients with myocardial infarction suggest that administration of RheothRx Injection together with thrombolytic therapy has beneficial effects on infarct size, left ventricular function and risk of death, reinfarction or shock (34). RheothRx Injection has been shown to have beneficial effects for the treatment of sickle cell disease (13); a pilot clinical study on patients with moderate to severe crisis pain showed that RheothRx Injection reduced the crisis duration by 32% and total analgesia requirement by 73% ($p = 0.097$ and 0.036 respectively) (13).

The aforementioned effects of poloxamer 188 have been attributed to both rheological and antithrombotic actions. Poloxamer 188 has been clearly shown to improve whole blood viscosity and t reduce both the extent and the strength of RBC aggregation *in vitro* at concentrations as low as 0.25mgmL^{-1} (6,35,36). Evidence for an antithrombotic effect comes from animal studies: a) poloxamer 188 prevented thrombus propagation and secondary platelet aggregation in a pig model of coronary artery thrombosis (12); b) in a dog model of copper-coil induced arterial thrombosis, poloxamer 188 reduced the time required for clot lysis with tissue-type plasminogen activator (tPA) (10). However, despite these reports of *in vivo* antithrombotic effects, *in vitro* studies have been unable to detect an effect of poloxamer 188 on platelets at clinically relevant plasma concentrations. For example, the effect of poloxamer 188 on *in vitro* platelet aggregation induced by exogenous ADP was investigated by Benner et al. (14), who showed that although high concentrations (up to 40mgmL^{-1}) of the copolymer caused a dose-dependent inhibition of platelet aggregation in PRP, no effect was observed at a copolymer concentration below 5mgmL^{-1}. As the maximum plasma concentration after intravenous administration is of the order of 1mgmL^{-1} (37), these results (14) are inconsistent with the observed *in vivo* antithrombotic effects of poloxamer 188. The present study appears to resolve this inconsistency. While no effect was detected herein for agonist-induced platelet aggregation in PRP (Fig. 3), these data demonstrate that poloxamer 188 is a potent inhibitor of RBC-induced platelet aggregation in whole blood, with 41% inhibition at a plasma copolymer concentration of 0.05mgmL^{-1} and almost complete inhibition at 1.0mgmL^{-1} (Table I). Therefore, using RBC-induced platelet aggregation as a test system, (which may more closely mimic *in vivo* situations e.g., conditions of turbulent flow within atherosclerotic vessels or prosthetic devices) as opposed to aggregation induced by the addition of exogenous stimuli, allows the inhibitory effects of poloxamer 188 to be demonstrated at plasma concentrations consistent with *in vivo* levels.

The mechanism by which platelets aggregate *in vitro* in agitated whole blood samples is believed to be due to microenvironment release of trace quantities of ADP from RBC (19,38-42). The findings presented in this study are consistent with this mechanism: The observation that platelet aggregation does not occur in agitated samples of PRP, and that platelet aggregation was restored after addition of packed RBC to the PRP, confirm the role of RBC. Moreover, the fact that the ADP-depleting system PEP/PK substantially blocked RBC-induced aggregation in this study strongly indicates that ADP leakage from mechanically stressed RBC is the cause of (or at least initiates) the platelet aggregation. However, poloxamer 188 does not directly interfere with the actions of ADP on platelets (Fig. 3) unlike PEP/PK or 2-ClAd, nor does poloxamer 188 interfere with the ability of platelets to adhere to each other after stimulation. Thus it is clear that poloxamer 188 inhibits RBC-induced aggregation by an entirely different mechanism, presumably via an interaction with RBC rather than directly with platelets.

Electrophoretic mobility and hydrophobic interaction chromatographic techniques (4,43) have shown that the adsorption of poloxamers to polystyrene nanospheres occurs via the polyoxypropylene core; the thickness of the adsorbed layer is related to the polyoxyethylene content of the copolymer, indicating that the hydrated polyoxyethylene blocks remain extended in the bulk phase. Thus the hydrophobic surface of the nanosphere is converted to a non-adhesive hydrated surface with flexible, hydrophilic polyoxyethylene chains extending into the surrounding medium. Poloxamer-coated polystyrene nanospheres show dramatically enhanced biocompatibility

in model drug delivery systems, as reflected by reduced sequestration by the liver and greatly prolonged circulatory times (4). RheothRx® Injection and related poloxamers have been shown to reduce osmotic fragility of RBC (44) and hemolysis of RBC during cardiopulmonary bypass (8), and to strongly inhibit RBC-RBC aggregation (6,14,35,36) and adhesion of RBC to endothelial cells (45). These effects are thought to be mediated through poloxamer interactions with the RBC surface, because poloxamers appear to adsorb non-specifically to cell membranes. We therefore propose that poloxamer 188 inhibits RBC-induced platelet aggregation in a similar manner: Non-specific adsorption of the copolymer to the RBC membrane occurs via the hydrophobic polyoxypropylene block, while the hydrated polyoxyethylene blocks remain extended in the bulk phase forming a cytoprotective "barrier" which prevents close interactions between adjacent RBC or between RBC and the vessel wall and thereby prevents damage to the RBC membrane. Consequently, ADP release from RBC is minimized and thus platelet aggregation is prevented. It is important to note that this mechanism is entirely different from other inhibitors of spontaneous platelet aggregation which act by depleting ADP (e.g., PEP/PK) or competitively inhibiting the effects of ADP (e.g., 2-ClAd) after it is released from sheared RBC.

The extent to which RBC-induced platelet aggregation plays an important role *in vivo* under normal conditions is unclear. However, RBC-induced platelet aggregation is likely to occur in situations where the endothelium is damaged or incomplete, or where there is no endothelium (e.g., after endarterectomy, or on prosthetic surfaces, including vascular grafts) (19,25). RBC-induced platelet activation also is common in patients with prosthetic heart valves and in patients undergoing bypass surgery with extracorporeal circulation (8). In many of these conditions, antiplatelet agents such as aspirin and dipyridamole are used with success to reduce the risk of thrombosis or re-thrombosis. Dipyridamole is of particular value for preventing platelet activation in the case of artificial heart valves and vascular prostheses (46), and is of interest because, like poloxamer 188, it has only a minimal effect on true agonist-induced platelet aggregation but does inhibit 'spontaneous' RBC-induced platelet aggregation at pharmacologically relevant doses (20). RheothRx Injection, therefore, would appear to have significant therapeutic potential for prevention of re-thrombosis after endarterectomy or insertion of vascular prostheses. Additional risk of hemorrhage seems unlikely, because poloxamer 188 does not interfere with normal platelet function, and poloxamer 188 has been shown to have no effect on bleeding time, prothrombin time or partial thromboplastin time *in vitro* or *ex vivo*. RheothRx (poloxamer 188) Injection has low toxicity (1,2) provided that the dose is adjusted for renal function (personal communication, M.K. Jolly, Burroughs Wellcome Co.), a half-life of approximately 5 hours after intravenous injection (37), is not metabolized, and has been demonstrated to be well tolerated in clinical studies on human volunteers at plasma concentrations up to 1.31mgmL^{-1} (13, 37).

SUMMARY

Although RheothRx Injection has minimal effects on platelet aggregation induced by agonists such as ADP, it is strong inhibitor of 'spontaneous' or RBC-induced platelet aggregation at clinically relevant concentrations. We propose that the inhibition of RBC-induced platelet aggregation by RheothRx (poloxamer 188) Injection is a consequence of non-specific adsorption of the copolymer to the RBC surface via the hydrophobic polyoxypropylene moiety, with the

polyoxyethylene blocks forming a cytoprotective sheath around the RBC. The cytoprotective effect of poloxamer 188 prevents RBC membrane damage due to cell-cell and cell-container surface collisions, and hence inhibits platelet activation and aggregation by prevention of ADP release. The inhibitory effects reported here represent the first observation of effects of poloxamer 188 on platelet aggregation at clinically achievable physiological concentrations (<1mgmL^{-1}). In view of its effectiveness and low toxicity, RheothRx® Injection appears to have potential as an antithrombotic agent for treatment in pathological conditions of the vascular system characterized by thrombosis, as well as prevention of RBC damage from vascular prostheses or extracorporeal circulation.

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1) Rubin et al., New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. Journal of Vascular Surgery, (1992) 15/4 (683-692).

2) Armstrong et al., Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection), THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.

3) ELY et al., PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA, CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904.

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Thanks a bunch,
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Research Advances Series

Protective Effects of Aden sine In Myocardial Ischemia

Stephen W. Ely, PhD, MD, and Robert M. Berne, MD

Adenosine functions in a multiplicity of physiological and pathophysiological ways and serves as a negative feedback regulator in the cardiovascular system as well as in some cell types. Many of the actions of adenosine are homeostatic and protective in nature, and the nucleoside has been termed a retaliatory metabolite[1] because of these properties. The present review is limited to the involvement of endogenous and exogenous adenosine in myocardial ischemia and the means whereby adenosine can protect the heart from the deleterious effects of an inadequate blood flow and oxygen supply.

Adenosine and Myocardial Reperfusion Injury

Myocardial Stunning

Recently, considerable attention has been focused on the pathophysiology of myocardial ischemia with subsequent reperfusion and on methods of reducing reperfusion injury and the associated reversible postischemic dysfunction of the ventricles.[2–9] The prolonged dysfunction of the ventricles after an episode of ischemia has been termed myocardial stunning, as contrasted to irreversible injury characteristic of myocardial infarction. Furthermore, there has been an enormous interest in the pharmacological modification of this phenomenon as a result of the technical advances in the areas of thrombolytic and angioplastic recanalization of stenotic or occluded coronary arteries, cardioplegic arrest during cardiac surgery, and organ preservation techniques for cardiac transplantation.

Stunned myocardium is characterized by metabolic and functional abnormalities that occur after a period of ischemia that may persist for hours to days. There are measurable defects in myocardial cell volume and ion ontent, loss of intracellular nucleotides, and contractile dysfunction.[2–8,10–13] There are many possible mechanisms of myocardial stunning.

Inability of the cell to produce sufficient energy. Adenosine triphosphate (ATP) levels are known to be depressed for hours to days after an ischemic episode.[3,11,12,14,15] During ischemia, mitochondrial function (as measured by the ratio of state 3 to state 4 respiration) remains intact while ATP levels fall significantly.[16] Reduced substrate in the form of adenosine monophosphate (AMP) or diphosphate (ADP) may play a role in myocardial stunning.[17] Furthermore, with an interruption of blood flow to the myocardium, there is a fall in tissue P_{O_2}, mitochondrial electron flow ceases, and there is a rapid mitochondrial ATPase-mediated hydrolysis of ATP. This hydrolysis of ATP is limited by the decrease in pH that develops during ischemia.[18]

Inability to use energy. Creatine kinase activity and free ADP are reduced in stunned myocardium and may contribute to functional abnormalities of the myofibrils.[19,29]

Inadequate myocardial perfusion. Factors that may contribute to an insufficient blood flow include endothelial and myocardial cell swelling, microvascular thrombosis, leukocyte plugging, and vascular smooth muscle dysfunction.[21–25]

Generation of oxygen free radicals from activated neutrophils and from the endothelium. Reversible ischemia may result in the formation of oxygen free radicals, including the superoxide anion (O_2^-), the hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2). These oxygen free radicals may contribute significantly to depressed myocardial function with reperfusion of ischemic myocardium. Free radicals are highly reactive and capable of causing membrane lipid peroxidation,[26,27] protein (enzyme) denaturation,[28] and sarcolemmal[29] and sarcoplasmic reticulum dysfunction.[30] Beneficial effects of antioxidants on stunned myocardium[31–33] support a role for free oxygen radicals in the damage observed in reperfused myocardium.[34–36]

Other hypotheses proposed for ventricular dysfunction in stunned myocardium include sarcoplasmic reticulum dysfunction with reduced availability of calcium to the contractile apparatus,[37] reduced calcium sensitivity of the contractile apparatus despite elevated intracellular calcium concentrations,[38] and calcium overload, which may be transient and may contribute to damage of intracellular organelles.[39] An extensive review of this topic recently appeared in this journal and the reader is referred to it for more detailed information.[40]

Summary. Myocardial dysfunction after an ischemic episode is multifactorial in etiology. Factors reported to be associated with myocardial stunning are high-energy phosphate production and utilization, inadequate myocardial perfusion, free radical injury, and alterations in calcium metabolism.

Regional Reperfusion Injury

The use of intra-arterial adenosine infusion to hasten recovery of regionally stunned myocardium was initially

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predicated on the fact that ischemia results in depletion of ATP.[41–43] If the oxygen deprivation lasted longer than 10 minutes, the repletion of ATP was a prolonged process.[3,11,12,14,15,44–47] This is attributed to very slow rates of d novo nucleotide synthesis[46–48] and to limited salvage of adenosine, inosine, and hypoxanthine because of their rapid washout from the tissue on reperfusion.[49,50] Regional ischemia is also associated with regional contractile dysfunction,[2–4,13] and a causal relation between ATP content and myocardial function has be n hypothesized,[14,51–53] although evidence exists both for and against such a relation.[54]

The efficacy of adenosine as a substrate for myocardial nucleotide formation was first demonstrated by Isselhard et al.[55,56] In these studies, adenosine was infused into the left atrium, left ventricle, or vena cava at rates up to 2–3 μmol/kg/min in rabbits, rats, and dogs. ATP levels in myocardial tissue were increased above the mean control value by 39% in rabbits, 48% in rats, and 21% in dogs. These changes occurred without a change in ventricular function.

In subsequent studies with a canine model of 45-minute occlusion of a diagonal branch of the left anterior descending (LAD) coronary artery and a 3-hour period of reperfusion, intracoronary infusion f radiolabeled adenosine was shown to increase ATP formation by 90-fold.[57] In a similar study, the increase in ATP was not associated with an improvement in regional contractile function.[58] Conversely, Wyatt et al[59] showed in a canine model of 15-minute LAD occlusion that an intracoronary infusion of adenosine (0.1 μmol/min) during the reperfusion period resulted in a marked improvement in segmental ventricular systolic shortening during the 2 hours of reperfusion studied. They reported a 68% recovery of shortening in the adenosine-treated group and a 17% recovery in the control group at 2 hours of reperfusion.[59] These findings have recently been confirmed in a similar model, and this study[60] also showed that the beneficial effects of adenosine on recovery of regional ventricular function could be prevented with the adenosine receptor antagonist 8-phenyltheophylline.

The relation between regional ATP concentration and postischemic myocardial dysfunction is complicated, controversial, and beyond the scope of this review. For example, inotropic agents are capable of stimulating the stunned myocardium without a decrease in ATP levels, suggesting that energy production can meet increased demands under these conditions.[19] Furthermore, interventions that increase ATP levels in the stunned heart have not been successful in restoring mechanical function.[61] However, infused adenosine does increase myocardial ATP formation during basal conditions and after ischemia. Furthermore, adenosine infusions result in a significant improvement in postischemic regional contractile function with brief (15 minutes) but not long (45 or more minutes) periods of coronary occlusion. It is unclear whether the beneficial effect of adenosine on ventricular function is related to changes in adenine nucleotide levels or whether this beneficial effect is derived from other ffects on myocardial metabolism (i.e., glycolysis), microvascular perfusion, or oth r undetermined mechanisms.

Infarct Size Reduction

A series of articles has recently been published by investigators at Vanderbilt University, Nashville, Tenn., on the ability of adenosine to limit infarct size in a canine model of LAD occlusion with reperfusion.[62–65] Olafsson et al[62] administered an intracoronary infusion of adenosine at a rate of 3.75 mg/min for the first hour of reperfusion after a 90-minute LAD occlusion. Infarct size, measured by vital staining with triphenyltetrazolium at 24 hours, was reduced in the adenosine-treated group, both when expressed as percent infarct of the area at risk (10% versus 41%) and as a percent of the left ventricle (4% versus 18%). Regional ventricular function was assessed by contrast ventriculography. Both regional and global indexes of ventricular function were substantially improved over control in the adenosine-treated group.[62] Furthermore, light and electron microscopy revealed that adenosine treatment reduced the degree of neutrophil infiltration and capillary plugging and enhanced endothelial preservation. These findings were confirmed in a study of similar design, but the duration of LAD occlusion was 120 minutes.[63] However, when the length of LAD occlusion was extended to 180 minutes, cardioprotective effects of adenosine were lost.[64] In a more recent study with this same model (90-minute LAD occlusion, 72-hour reperfusion), adenosine (0.15 mg/kg/min) was infused intravenously during the first hour of reperfusion. There were no significant effects on heart rate or blood pressure, and infarct size (as percentage of area at risk) was 35% in controls versus 17% in the adenosine-treated group. Better regional ventricular function, reduced capillary plugging, and preservation of endothelial cell structure were all seen in the adenosine-treated group.[65]

Studies on adenosine and infarct size reduction are not all in agreement. Homeister et al[66] reported that in a canine model with 90 minutes of left circumflex coronary artery occlusion and 6 hours of reperfusion, intracoronary adenosine infusions that yielded calculated blood concentrations of 200–600 μmol/l had no effect on infarct size unless the adenosine was administered in combination with lidocaine (2.0 mg/kg i.v.). It is also noteworthy that in the previously cited studies,[62–65] lidocaine was administered at the time of occlusion and at the onset of reperfusion.

Retrograde coronary venous infusion of adenosine has also been reported to reduce infarct size.[67] In this study, adenosine (20 μg/kg/min) was infused retrogradely via the great cardiac vein for 30 minutes after a 60-minute occlusion of the LAD coronary artery in pigs. Lidocaine was not given in this study. Retrograde adenosine infusion resulted in an infarct size of 27% of the area at risk, whereas right atrial infusion and vehicle controls resulted in infarct sizes of 62% and 56% of the area at risk, respectively.

Summary. Intracoronary infusion, retrograde coronary venous infusion, or intravenous infusion of adenosine can significantly reduce infarct size if the length of occlusion is less than 3 hours. Lidocaine may confer additional cardioprotection when used in combination with adenosine.

Global Ischemic Injury

Open heart surgery requires a period f aortic cross-clamping during cardiopulmonary bypass. The degree of myocardial injury during these episodes of global ischemia is markedly reduced with standard cardioplegic techniques of hypothermia and potassium-induced arrest. However, these methods are not wholly efficacious because metabolic and functional injury secondary to ischemia and reperfusion have been documented both in experimental and clinical models.[68–73] Whereas there may be many mechanisms involved in reperfusion injury,[3,11,12,14–40] a defect in metabolism plays a significant role.

Benson et al[74] first suggested that recovery of ATP during reperfusion is limited by a lack of nucleotide precursors that are degraded and washed out with ischemia and subsequent reperfusion.[75] This concept led to an ever-growing interest in substrate enhancement of postischemic myocardium. Research efforts have since focused on a multitude of substrates, the discussion of which is beyond the scope of this review but can be found elsewhere.[76]

A number of investigators have provided evidence for the use of adenosine as a substrate for ATP resynthesis during global ischemia and reperfusion. Reibel and Rovetto[77,78] conducted studies in which isolated rat hearts were exposed to low-flow ischemia (60% reduction in coronary flow) until ventricular failure occurred and then for an additional 30 minutes. The hearts were then reperfused for 30 minutes at normal flow rates. Adenosine (50 μmol/l) was added to the perfusion fluid at the time of onset of ischemia and maintained throughout reperfusion. This procedure had no effect on tissue high-energy phosphate concentrations. However, a close relation between ATP concentrations and ventricular power in postischemic hearts was noted,[77] but this does not indicate a cause-and-effect relation. In a second study, these authors[78] extended the period of reperfusion with 50 μmol/l radiolabeled adenosine from 30 to 300 minutes. After 5 hours of reperfusion with adenosine, tissue ATP levels recovered from a level of 50% of control values at the onset of reperfusion to ~100% by 300 minutes of reperfusion, whereas ATP levels in the group reperfused without adenosine failed to recover over 300 minutes of reperfusion.

Our laboratory observed the direct effects of adenosine on adenine nucleotide concentrations and ventricular function in an isolated perfused rat heart preparation in which heart rate, ventricular preload and afterload, and coronary flow rate were all held constant.[79] These hearts were perfused with or without 100 μmol/l adenosine throughout the protocol of 30 minutes of equilibration, 10 minutes of normothermic ischemia, and 60 minutes of reperfusion. Adenosine significantly increased ATP levels at the end of the ischemic period and during the reperfusion phase. Ventricular function was increased during the reperfusion period in the adenosine-treated group; however, these data do not establish a cause-and-effect relation.

Summary. ATP depletion can be retarded by adenosine, and this effect is associated with improved postischemic ventricular function, thus supporting the use of adenosine as a cardioprotective agent in global ischemia. Whether this effect is mediated via a salvage mechanism or through other receptor-mediated mechanisms has yet to be ascertained.

Cardioplegia

In an initial study addressing the cardioplegic potential of adenosine, F ker et al[80] used a canine model of global normothermic ischemia for 20 minutes while the animals were supported on cardiopulmonary bypass. In addition to adenosine alone (20 mg/kg i.a. bolus injection), the adenosine deaminase inhibitor EHNA (erythro-9-[2-hydroxy-3-nonyl]adenine hydrochloride) was also used alone (10 mg/kg) or in combination with adenosine during 20 minutes of ischemia and 30 minutes of reperfusion. Adenosine or EHNA alone had no effect on reperfusion ATP concentrations. However, EHNA plus adenosine restored ATP to 88% of the preischemic ATP levels. Silverman et al[81] used hypothermic, potassium-arrested dog hearts in situ and a higher dose of adenosine for aortic root infusion during reperfusion (40 mg/kg). They found that after 60 minutes of ischemia, adenosine alone or in combination with EHNA (10 mg/kg) maintained ATP at a level no different from preischemic levels over a 60-minute reperfusion period. Hence, adenosine prevented the fall in ATP normally seen during reperfusion. However, this study did not evaluate ventricular function.

In a study in which heart rate, preload, afterload, and coronary blood flow were held constant in an in vivo canine model subjected to 1 hour of global normothermic ischemia,[82] our laboratory compared standard hyperkalemic cardioplegia with purine-enriched asanguinous cardioplegia (adenosine 100 μmol/l, hypoxanthine 100 μmol/l, and ribose 2 mmol/l)[82] or blood cardioplegia.[83] In the purine-enriched cardioplegia group, ATP degradation during ischemia was significantly reduced, and postischemic recovery of ventricular function was significantly improved when compared with standard asanguinous or blood cardioplegia.

Reports of the optimal dose of adenosine in cardioplegic solutions of the isolated heart model vary. Hohlfeld et al[84] evaluated adenosine infusions over a range of concentrations from 0 to 120 μmol/l and found that 15–30 μmol/l concentrations resulted in the greatest increment in baseline ATP levels (53%). However, the addition of 15 μmol/l adenosine to the perfusion fluid did not significantly improve contractile recovery after 20 minutes of normothermic ischemia and 45 minutes of reperfusion, suggesting that ATP and contractile recovery may not be related. Higher doses of adenosine, from 100 to 400 μmol/l, demonstrate beneficial dose-dependent effects on both adenine nucleotide preservation and postischemic ventricular function with an optimal concentration at ~200 μmol/l.[85–87] Several studies in both isolated crystalloid-perfused rat hearts and blood-perfused baboon hearts (in vivo) have demonstrated enhanced cardioprotection with adenosine with doses of 1–10 mmol/l.[88–91]

The use f adenosine as the sole cardioplegic agent[89] or in combination with potassium[90] is effective when used in high concentrations (1–10 mmol/l) by virtue of the ability of the nucleoside to induce cardiac arrest or to hasten potassium-induced arrest. These effects are caused by an adenosine-induced activation of the outward potassium current and subsequent membrane

hyperpolarization.[92] Furthermore, adenosine-supplemented cardioplegia is also effective at clinically relevant hypothermic temperatures.[88,89,93,94]

Summary. These studies suggest that adenosine, either alone or in combination with potassium cardioplegia, provides additional cardioprotection during normothermic or hypothermic global ischemia. Adenosine has been shown to reduce ATP degradation during ischemia, to increase ATP resynthesis during reperfusion, and to enhance postischemic ventricular function in crystalloid-perfused and blood-perfused hearts.

Cardiac Transplantation

Hyperkalemic arrest and/or hypothermic storage are currently the accepted methods for in vivo preservation of cardiac homografts and provide adequate protection for up to 4 hours.[95,96] Prolonged storage has been reported to result in tissue edema, a rise in coronary vascular resistance after transplantation, and vascular endothelial injury attributed to hyperkalemic storage solutions.[97–99] The ability to extend storage time and preserve transplant organ function has been the subject of many studies.

Recently, the use of adenosine in cardiac transplant preservation has gained attention. Petsikas et al[100,101] reported that the addition of adenosine (20 μmol/l) or AMP (0.1 mmol/l) to the Krebs-Henseleit perfusion fluid was successful in markedly preserving ventricular function in canine hearts that were stored for 24 hours in a continuous hypothermic perfusion system. Similar results have been reported by Ledingham et al.[94]

We have also found that adenosine increases the tolerance to ischemia in an isolated normothermic rat heart model.[79] The preischemic perfusion of 100 μmol/l adenosine for 30 minutes before the onset of total ischemia extended the time to onset of ischemic contracture by 50% and was associated with a reduced rate of ATP degradation.[79]

Summary. Adenosine prolongs the ischemic interval to irreversible contracture and preserves ventricular function after a storage period of up to 24 hours of hypothermic perfusion. Although there are no published studies evaluating adenosine effects on the function of the transplanted heart, further investigation is warranted to evaluate its use in transplant preservation.

Preconditioning

Murry et al[102] observed that myocardium that is first exposed to multiple brief periods of ischemia is more tolerant to a subsequent episode of prolonged ischemia. They termed this phenomenon ischemic preconditioning. Their data indicate that infarct size in response to a 40-minute coronary occlusion was reduced by 75% (29–7% of area at risk) when the area supplied by the occluded artery had been previously subjected to four successive 5-minute periods of ischemia, each separated by 5 minutes of reperfusion. Preconditioning dramatically increased the tolerance of the myocardium at risk to an therwise lethal ischemic event. The increased tolerance to ischemia is associated with a reduced rate of energy utilization during ischemia and a slowing of glycolysis and glycogenolysis.[103] This effect of preconditioning cannot be explained on the basis of metabolic depression derived from postischemic contractile dysfunction.[104]

Preconditioning has been reported in dogs,[102] rabbits,[105] and pigs.[106] Also, it has been suggested that a preconditioning effect could be conferred in humans by sequential 90-second occlusions in patients undergoing elective coronary angioplasty.[107] The mechanism responsible for preconditioning is not completely known; other mechanisms in addition to the observed reduction of energy utilization may be involved.[103] Recently, adenosine has been implicated in preconditioning,[108] and the beneficial effects of preconditioning could be prevented by pretreatment with the adenosine receptor antagonist 8-sulfophenyltheophylline. This observation suggests that endogenous adenosine is involved in the preconditioning effect.[108] Furthermore, intravenous or intracoronary adenosine or the selective α_1-receptor agonist PIA (N^6-phenylisopropyl adenosine) instead of the ischemic preconditioning protocol conferred a similar protective effect against the subsequent prolonged ischemic episode.[109,110] These observations suggest that adenosine α_1-receptor activation may be involved in the protective changes seen in preconditioning.

Summary. Intravenous or intracoronary infusion of adenosine or the α_1-receptor agonist PIA confers a protective effect on the myocardium similar to a preconditioning protocol, and the preconditioning effect can be prevented by adenosine receptor antagonists.

Cardioprotective Actions by Adenosine

Formation and Metabolism

It is clear that protection of the heart from episodes of ischemia or hypoxia can be achieved with both endogenous and exogenous adenosine. Endogenous adenosine is primarily formed from the dephosphorylation of AMP that may occur intracellularly and extracellularly; 5′-nucleotidase (both membrane bound and cytosolic) dephosphorylates AMP to adenosine.[111] AMP is derived from cytosolic ATP and ADP and cAMP, or from extracellular sources of nucleotides such as blood elements,[112] endothelium,[113,114] and adrenergic nerves.[115] However, the major source of coronary adenosine is the cardiomyocyte[116] by the action of cytosolic 5′-nucleotidase.

Adenosine can also arise from the hydrolysis of *S*-adenosylhomocysteine (SAH) to adenosine and homocysteine, a reaction catalyzed by SAH hydrolase. The equilibrium constant favors the formation of SAH, and a large percentage of the intracellular adenosine is bound to SAH and cannot be degraded by adenosine deaminase. Furthermore, adenosine release from SAH hydrolysis does not increase during hypoxia-induced increases in cardiac adenosine formation and release.[117]

Endogenous or exogenous adenosine is removed by 1) phosphorylation by adenosine kinase to AMP, 2) degradation to inosine by adenosine deaminase, or 3) washout in the circulation. Because the K_m for adenosine kinase is 100-fold lower than that for adenosine deaminase,[118] the preferential pathway for adenosine metabolism is for salvage by phosphorylation to AMP. The purine salvage pathway is critical for the regeneration of cardiomyocyte adenine nucleotide pools after an ischemic or hypoxic episode because the pathway for

de novo nucleotide synthesis accounts for only 0.4% f the total nucleotid pool per hour.[47] Purine salvage can also be accomplished by the degradation f adenosine to inosine, which is then degraded to hypoxanthine, which can be phosphorylated to IMP and th n aminated to AMP. Furthermore, adenosine can be converted to adenine, which can be ribosylated to form AMP. Both of these secondary salvage pathways (for hypoxanthine and adenine) are limited because they require phosphoribosylpyrophosphate, which is present in low concentrations in the myocardium.[119,120] However, adenine[121] and hypoxanthine[122] have been shown to be effective in preserving postischemic ATP and myocardial function.

Therefore, the purine salvage pathways represent the primary mechanism whereby endogenous or exogenous adenosine (and its metabolites) contribute to the preservation of the adenine nucleotide pool during periods of oxygen deprivation and reperfusion.[78,123–127]

Transport and Deaminase Inhibitors

Adenosine and other nucleosides traverse the cell membrane via a nucleoside carrier system that represents a reversible process of simple and facilitated diffusion.[128] Several agents are known to inhibit the nucleoside transporter and therefore might theoretically keep nucleosides sequestered in the cell to promote the maintenance of adenine nucleotide levels during ischemia and reperfusion. These agents include dipyridamole, mioflazine, and NBMPR (nitrobenzylmercaptopurine riboside).

Dipyridamole has been shown to inhibit nucleoside (adenosine) transport,[129,130] and several studies have demonstrated its ability to reduce infarct size,[131–133] although not all studies are in agreement.[134]

Mioflazine is a chemical analogue of lidoflazine and also inhibits the nucleoside transporter.[135] Mioflazine has been shown to reduce the release of adenosine[136] and preserve high-energy phosphates and ventricular function after an ischemic insult.[137] Both dipyridamole and mioflazine increase coronary blood flow[131,138] as well as coronary collateral blood flow, which may aid in the reduction of infarct size.[131] In addition to inhibiting the nucleoside transporter, mioflazine also is a calcium channel antagonist that may contribute to its cardioprotective properties. In a recent clinical study, Rosseau et al[139] reported that patients treated with dipyridamole after thrombolysis had better indexes of ventricular relaxation and contraction. These preliminary data suggest that adenosine transport inhibition might improve ventricular function after thrombolysis-induced reperfusion. However, these findings await further study and confirmation.

Dipyridamole is also used to assess coronary perfusion in conjunction with myocardial ^{201}Tl scanning in patients as a noninvasive test to assess coronary artery disease. Dipyridamole is believed to dilate coronary vessels indirectly, with its mechanism of action involving local increases in adenosine concentration by adenosine transport inhibition. Dipyridamole[140] and, to a lesser extent, ad nosine infusions[141] have be n shown to decrease the subendocardial-to-subepicardial flow ratio, and in the case of dipyridamole,[140] endocardial flow has been shown to decrease, implicating a true steal phenomenon. This suggests that dipyridam le may induce subendocardial ischemia in the presence of significant coronary stenosis. Transient myocardial ischemia with regional contractile dysfunction, chest pain, and ischemic electrocardiographic changes has been reported in patients with isolated stenoses of the LAD coronary artery in response to dipyridamole.[142]

It is also interesting to note that coronary ischemia does not result in maximal vasodilation. It has been suggested that local or reflex α-adrenergic tone may limit ischemia-induced vasodilation.[143,144] Vasodilators such as adenosine have been shown to provide additional vasodilation when infused during myocardial ischemia.[141]

The nucleoside transport blocker NBMPR has also been shown to increase cellular adenosine during ischemia and to enhance ATP repletion during reperfusion.[145] This drug, when combined with the adenosine deaminase inhibitor EHNA, markedly improves postischemic ventricular function.[146]

Inhibitors of adenosine deaminase such as EHNA or 2-deoxycoformycin increase levels of myocardial adenosine and reduce its breakdown to inosine and hypoxanthine.[147] Adenosine deaminase inhibitors in the setting of myocardial ischemia and reperfusion increase tissue and interstitial adenosine levels, promote ATP restoration, and improve postischemic ventricular function.[86,148–152] These effects may be mediated by enhanced purine salvage and by reduced free radical–induced injury.[145,146] Because uric acid also acts as a free radical scavenger, limiting its formation with the use of adenosine deaminase inhibitors could also paradoxically enhance free radical–induced injury. The overall effects of adenosine deaminase inhibition on free radical–induced injury have not been studied.

Receptors

Adenosine receptors are classified as A_1, those that inhibit adenylyl cyclase, and A_2, those that stimulate this enzyme system.[153] These receptors have been characterized on the basis of radioligand binding studies and the specific pharmacological responses to adenosine and its analogues. In the heart, A_1-receptors are found on cardiomyocytes and vascular smooth muscle, whereas A_2-receptors are found on endothelium and vascular smooth muscle. Cardiac cell A_1-receptors mediate the negative chronotropic,[154] dromotropic,[154] and inotropic[155] responses. A_1-Receptors in the heart also appear to mediate the beneficial effects of adenosine in prolonging the time to ischemic contracture[156] and preconditioning,[109,110] which may involve activation of G_i proteins[157] and glycolytic flux.

Adenosine A_2-receptors are apparently located on the coronary vessels, because intravascular A_2-agonists are more potent coronary vasodilators than A_1-agonists.[158] Thus, it has been postulated that exogenous adenosine stimulates A_2-receptors, which induce vascular smooth muscle relaxation, although the mechanism for this response has not yet been elucidated. Endothelial receptor stimulation by exogenous adenosine in guinea pig aorta contributes approximately 30% of the vasodilati n. The remaining 70% results from direct stimulation f receptors on vascular smooth muscle and is observed after removal of the endothelium.[159]

Therefore, adenosine and its analogues aid in protection of the heart from injurious effects of ischemia and hypoxia by activation of the adenosine receptors.

Coronary Blood Flow

The earliest response to an inadequate blood supply to the myocardium appears to be dilation f the coronary resistance vessels. Considerable evidence supports the concept that ischemia or hypoxia-induced vasodilation is mediated by the release of adenosine from the myocardial cells.[160–162] Furthermore, the degree of vasodilation and the release of adenosine from the heart are directly proportional to the degree of oxygen deprivation.[163] Adenosine represents the first line of defense in protection of the myocardium against a decrease in the oxygen supply/demand ratio[164,165] by reducing coronary resistance and maximizing coronary blood flow.

Atrioventricular Conduction and Excitation

The difference in the sensitivity of the atrioventricular (AV) node and the coronary vascular smooth muscle to endogenous adenosine produced by hypoxia is that when the coronary resistance vessels become maximally dilated, a greater degree of oxygen deprivation elicits a greater production of adenosine. The resulting higher myocardial adenosine concentration produces AV conduction delay or block, which represents another mechanism to rectify the oxygen supply/demand imbalance. This dromotropic effect, which has been studied extensively in the isolated perfused guinea pig heart, is also mediated by adenosine.[166] A moderate decrease in the global oxygen supply of the isolated perfused guinea pig heart elicits a conduction delay between the atrium and the bundle of His, and a greater reduction in oxygen supply results in AV block.[167] The degree of impairment of AV conduction is roughly proportional to the adenosine formed in the hypoxic or ischemic myocardium.[166,167]

Conduction delay or block is a result of activation of the A_1-adenosine receptor[167] and is attenuated or abolished by administrations of adenosine receptor antagonists or adenosine deaminase[167] and potentiated by adenosine transport blockers, which increase interstitial fluid adenosine levels by blocking uptake of the nucleoside.[167] Adenosine has been shown to act similarly on the human AV node[168]; in fact, it is used clinically to transiently block AV conduction as a means of terminating supraventricular tachycardia in which the AV node is part of the reentrant pathway.[168] By producing AV block, the heart rate is decreased, which reduces the oxygen needs of the heart, thereby aiding in protection f the myocardium from the detrimental effects of oxygen deprivation.

With high concentrations of myocardial adenosine, which can occur with severe reductions in oxygen delivery, the sinoatrial (SA) node becomes depressed via activation of A_1-receptors. The resulting bradycardia reduces the oxygen requirements of the heart further, thus affording additional protection against ischemic damage to the myocardium. This effect of adenosine on the SA node observed in guinea pig hearts[154] has also been demonstrat d in humans[168] and can be abolished by A_1-receptor antagonists and potentiated by adenosine uptake blockers.[167] Furthermore, other pacemaker cells (e.g., His bundle, Purkinje fiber) are also depressed by adenosine.[167]

Inotropy

In the atria, adenosine has a direct negative inotropic effect.[169] It shortens or abolishes the action potential[170] and causes hyperpolarization of the membrane by enhancing potassium efflux by activation of the potassium channels.[92,167] However, in ventricular muscle, a direct effect is lacking and a negative inotropic response is observed only if the tissue is first stimulated by catecholamines and the adenylyl cyclase system is activated.[171,172] As with the chronotropic and dromotropic effects on the heart, adenosine activates the membrane A_1-receptors, and the elicited responses are attenuated by adenosine deaminase and adenosine antagonists and potentiated by adenosine transport blockers and adenosine deaminase inhibitors. Studies with perfused hearts[172] and membranes prepared from ventricular myocardium[172,173] indicate that adenosine attenuates the β-adrenergic–enhanced adenylyl cyclase activity but not the basal activity of this enzyme. Furthermore, the A_1-adenosine agonist phenylisopropyladenosine attenuates the β-adrenergic-induced increase in adenylyl cyclase by its action on signal transduction of the β-receptor.[173] Adenosine also inhibits the release of norepinephrine from stimulated sympathetic nerve fibers in the heart; short periods of myocardial ischemia release sufficient adenosine to significantly reduce the neural release of norepinephrine.[174] Myocardial ischemia results in the release of endogenous catecholamines,[175] which stimulate myocardial metabolism and increase oxygen needs. Ischemia also results in the production and release of adenosine.[160]

Therefore, in ischemia, endogenous adenosine can function in a protective manner by decreasing the release of the metabolic stimulant norepinephrine and by attenuating the stimulating effect of the norepinephrine that is released.

Glycolysis

The metabolic response of the heart to hypoxia or ischemia involves an initial increase in glucose uptake and utilization, which helps in the preservation of myocardial ATP by glycolysis.[176] The rate-limiting step in the glycolytic pathway is the enzyme phosphofructokinase, which is stimulated by ADP, AMP, and inorganic phosphate, all of which are increased when oxygen availability is limited. Adenosine production is similarly increased in response to adenine nucleotide degradation, and a possible role for adenosine in mediating glucose transport has been proposed.[177] Raberger et al[177] demonstrated that intracoronary adenosine infusion in dogs increased myocardial glucose uptake without eliciting a concomitant change in oxygen utilization, although coronary blood flow was increased. Because coronary flow was not controlled and the increased glucose uptake seen during the infusion of adenosine may have been secondary to increas d substrate delivery, Turnheim et al[178] used the potassium-arrested isolated cat heart and compared constant pressure and constant flow perfusion. Adenosine infusion during constant pressure perfusi n (and increased coronary flow) resulted in an increase in glucose uptake

and utilization, whereas this effect failed to occur when coronary flow was held constant.[178] These results conflict with reports that adenosine increases glucose uptake in isolated hearts with both constant pressure[179] and constant flow perfusion.[179,180] Furthermore, Jesmok et al[181] demonstrated that adenosine increased glucose uptake in an isolated supported dog heart perfused at constant flow and that the effect was greater with increases in $M\dot{V}O_2$. This apparent relation of glucose uptake to $M\dot{V}O_2$ could explain the negative effects reported by Turnheim et al[178] in the potassium-arrested heart. More recently, Law and Raymond[182] observed that adenosine increased insulin-dependent glucose uptake in vivo in a euglycemic clamped dog heart and that the effect was not related to a change in $M\dot{V}O_2$ or coronary blood flow.

In a study in which glycolytic flux was measured, Wyatt et al[183] used an isolated rat heart model perfused at constant flow and demonstrated that adenosine produced a dose-dependent increase in glycolytic flux over a range of 25–100 μmol/l and that this effect was mediated by A_1-adenosine receptor stimulation. Furthermore, the increase in glycolytic flux in response to hypoxia or exogenous adenosine could be abrogated with the adenosine receptor antagonist 8-sulfophenyltheophylline.[183] Therefore, these studies generally support the hypothesis that adenosine production during hypoxia or ischemia may increase energy production through enhanced glucose transport. What effect this has on cell viability has yet to be defined.

Microvascular Injury

The vascular injury and resulting myocardial damage that occur in ischemia and reperfusion consist of a complex series of related events in which sequence and interaction are not fully understood.

With hypoxia or ischemia, myocardial perfusion can be reduced by a vasoconstrictor substance released by the endothelium[184] or by impairment of endothelium-mediated vessel relaxation by endothelium-derived relaxing factor (EDRF) caused by the action of superoxide anions released from activated neutrophils.[185] In crystalloid-perfused rat hearts, ischemia or hypoxia depressed endothelium-mediated dilation on reperfusion only if the reperfusion solution contained oxygen.[186] Furthermore, perfusion with buffer equilibrated with 95% N_2–5% CO_2 or containing superoxide dismutase prevented endothelial dysfunction. This observation plus the finding of elevated levels of superoxide anions in the cardiac effluent on reperfusion with oxygenated perfusion fluid indicates a cardiac source of the oxygen-derived free radicals.[186] An endothelium-independent injury is observed in the microvasculature as indicated by the blunted dilator response to papaverine during reperfusion after 2 hours of coronary occlusi n.[63] Endogenous adenosine from the myocytes released during ischemia probably prevents more extensive damage (than might occur if adenosine were not released) by inducing some d gree of relaxation of the vascular smooth muscle and protection of the endothelium and the vascular smooth muscle against the toxic effects of oxygen-derived free radicals. However, toxic effects of the ischemia and reperfusion override any beneficial effects of endogenously produced adenosine. Hence, higher concentrations of administered adenosine are required to combat the detrimental microvascular and parenchymal effects of ischemia.

A second mechanism whereby ischemia can produce microvascular damage and the related no-reflow phenomenon is by the plugging of capillaries with neutrophils. Engler and colleagues[187,188] have clearly demonstrated that the no-reflow response after periods of ischemia can be markedly attenuated or abolished if the leukocytes are removed from the blood. These observations are consonant with those of Cronstein et al,[189] who demonstrated that adenosine, acting via an A_1-adenosine receptor on the neutrophil, facilitates the chemotaxis of activated neutrophils. This enhanced chemotactic effect produced by adenosine could contribute to the obstruction of the capillaries by increasing the number of neutrophils in the ischemic region. To compound this injurious effect, activated neutrophils also release superoxide anions that cause considerable local vascular and tissue damage.[35,189,190] In contrast to the A_1-mediated effect of adenosine on neutrophils, A_2-adenosine receptor activation prevents adherence of the neutrophils to the vascular endothelium and also prevents the release of superoxide anions from the activated neutrophils.[189,191] The neutrophil A_1-receptor stimulation occurs at much lower concentrations of adenosine than does the A_2-receptor stimulation.[189] Hence, the endogenous adenosine concentration may be sufficient to promote neutrophil chemotaxis but not sufficient to inhibit superoxide anion release. However, the addition of pharmacological concentrations of adenosine during ischemia and/or reperfusion can prevent free radical release and thereby counter the neutrophil-mediated injury of the endothelium and myocardium.

Another factor that contributes to the microvascular damage and reduced tissue perfusion is the adherence of platelets to injured endothelial cells and aggregation of the platelets at these sites. Platelets release ADP, which enhances aggregation, and thromboxane A_2, which causes thrombus formation and local vasoconstriction. Release of 5-hydroxytryptamine from the platelets also contributes to the vasoconstriction. Adenosine formed in the ischemic myocardium (as well as some contributed by release from platelets)[192] opposes platelet aggregation and prevents microthrombosis.[193] This effect of adenosine represents another protective mechanism for the microcirculation and parenchymal tissue. However, exogenous adenosine provides additional protection because of the washout of endogenous adenosine during reperfusion.

Angiogenesis

Prolonged ischemia leads to irreversible myocardial damage, necrosis, and subsequent scarring. However, chronic myocardial ischemia as occurs with coronary artery stenosis leads to the development of collateral circulation. This type of growth of new blood vessels has been demonstrated in heart and skeletal muscle[194,195] and results from a reduction in the oxygen supply/demand ratio. Adair et al[196] have shown that adenosine is involved in this angiogenic effect of reduced oxygen supply. In support f this concept, it has been demonstrated that low oxygen concentrations (2%) increased proliferation of endothelial cells in culture and that culture media

from hypoxic cells stimulated proliferation of cells grown in 20% oxygen.[196] Addition of an adenosine receptor antagonist to the hypoxic conditioned media prevented this increase in endothelial cell proliferation. Furthermore, dipyridamole, an adenosine transport blocker that produces elevation of tissue adenosine levels, increased the formation of new capillaries in the rat heart.[197,198] These observations suggest that adenosine serves as a pr tective agent against prolonged periods of suboptimal blood flow by stimulating the development of new blood vessels. To what extent these observations can be extrapolated to humans remains to be explored. Nevertheless, adenosine may play a role in the development of the coronary collateral circulation frequently seen with coronary artery stenosis.

Summary

Adenosine is released from the myocardium in response to a decrease in the oxygen supply/demand ratio, as is seen in myocardial ischemia; its protective role is manifested by coronary and collateral vessel vasodilation that increase oxygen supply and by multiple effects that act in concert to decrease myocardial oxygen demand (i.e., negative inotropism, chronotropism, and dromotropism). During periods of oxygen deprivation, adenosine enhances energy production via increased glycolytic flux and can act as a substrate for purine salvage to restore cellular energy charge during reperfusion.

Adenosine limits the degree of vascular injury during ischemia and reperfusion by inhibition of oxygen radical release from activated neutrophils, thereby preventing endothelial cell damage, and by inhibition of platelet aggregation. These effects help to preserve endothelial cell function and microvascular perfusion. Long-term exposure to adenosine may also induce cor nary angiogenesis.

Conclusions

Endogenous adenosine plays a multifaceted role in protection of the ischemic myocardium. The pharmacological use of adenosine, its analogues, or its transport and metabolic inhibitors may extend its clinical application beyond its approved use as an agent for the termination of supraventricular tachyarrhythmias and provide significant new advances in myocardial protection in regional reperfusion, cardioplegic solutions, and preservation solutions for heart transplantation.

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KEY WORDS • reperfusion • cardioplegia • adenosine • glycolysis • endothelium • platelets • neutrophils • Research Advances Series • angiogenesis • myocardial stunning

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Please provide a copy of the following literature ASAP:

1) Rubin et al., New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. Journal of Vascular Surgery, (1992) 15/4 (683-692).

2) Armstrong et al., Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection), THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.

3) ELY et al., PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA, CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904.

4) Bastida et al., Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin, and phospholipase. Cancer Research, (1982) 42/11 (4348-4352).

5) Lee et al., In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency. BLOOD, (1979 Mar) 53 (3) 465-71.

6) Cattaneo et al., Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. BLOOD, (1990 Mar 1) 75 (5) 1081-6.

7) Wang et al., Exogenous adenosine application inhibits thrombus formation in stenosed canine coronary artery and partially protects against renewal of thrombus formation by epinephrine. FASEB Journal, (1995) Vol. 9, No. 3, pp. A322.

Thanks a bunch,
Gailene R. Gabel
7B15
305-0807

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Differentiation of Platelet-aggregating Effects of Human Tumor Cell Lines Based on Inhibition Studies with Apyrase, Hirudin, and Phospholipase[1]

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ABSTRACT

Three different mechanisms have been detected for the aggregation of platelets by tumor cells in a homologous human system based on inhibition studies with apyrase, hirudin, and phospholipase D. In the major group, platelet aggregation induced by the SKBR3 (adenocarcinoma), SKNMC (neuroblastoma), HT29 (adenocarcinoma), and HT144 (melanoma) cell lines was inhibited by apyrase and phospholipase D but not by hirudin, suggesting that adenosine 5′-diphosphate is involved in the first step. However, since the reaction occurs only in heparinized plasma, the mechanism must differ from that of platelet aggregation which can be induced in citrated platelet-rich plasma by endogenous or exogenous adenosine 5′-diphosphate. In contrast, the Hut28 (mesothelioma) line was inhibited by hirudin and phospholipase D but not by apyrase, suggesting that the mechanism in this system involves the activation of the clotting system in the early stages. However, the coagulant-dependent mechanism observed with Hut28 can be differentiated from the similar mechanism we have observed previously with the U87MG (glioblastoma) cell line since the latter is unaffected by phospholipase D (Am J. Hematol., *11*: 367–378, 1981). Phospholipase C had no effect on platelet aggregation induced by any of the human cell lines examined while both phospholipase A_2 and lysolecithin inhibited aggregation in every case. These results suggest that two categories of human tumor cells can be defined based on whether they initiate platelet aggregation by adenosine 5′-diphosphate or coagulant-dependent mechanisms. However, within this latter category, subclassification is possible based on the inhibitory effects of phospholipase D.

INTRODUCTION

Platelets in plasma can be aggregated by a wide variety of low-molecular-weight agonists such as ADP and epinephrine or by protein agonists such as thrombin and collagen. The mechanisms of these reactions are not clearly understood. A third class of interaction involves platelet aggregation induced by tumor cells (for review, see Ref. 7). Qualitative observations of interactions between platelets and tumor cells were first made during postmortem examination over a century ago and have been confirmed numerous times in a wide variety of model systems. In 1962, Gasic and Gasic (9) observed that the incidence of tumor metastases in mice given injections of mammary adenocarcinoma TA3 tumor cells was decreased if there was a prior injection of neuraminidase, an effect they ascribed to increased "stickiness" resulting from the action of neuraminidase on the membrane glycoproteins of the tumor cells or of host endothelial cells. Subsequently, they mad the important quantitative observation that this effect was due to neuraminidase-induced thrombocytopenia and that the incidence of lung tumors following injection of TA3 ascites cells was inversely proportional to the platelet count (11). They then extended these observations to show that there was a rough correlation between the abillty of various tumor cells to aggregate platelets *in vitro*, the number of lung metastases produced in mice, and the beneficial effect of thrombocytopenia in reducing metastasis (10).

Because of the difficulty of using *in vivo* assays for the quantitative assessment of platelet-tumor cell interactions, most subsequent studies have utllized aggregometry in attempts to elucidate the basic mechanisms involved (12–14, 17). Surface components of the tumor cell are thought to play a major role in initiating platelet activation. Rous virus-transformed rat kidney cells produce membrane vesicles in larger amounts than do untransformed cells, and these vesicles can cause platelet aggregation and release (8). A platelet-aggregating material has been extracted with 1 M urea from SV40-transformed mouse 3T3 fibroblasts (17), and in variants of th PW20 rat renal sarcoma line, correlations have been made between the production of the platelet-aggregating material, its sialic acid content, the ability of the tumor cells to induce platelet aggregation, and their content of cell surface sialic acid (18). The mechanism by which these cell surface compon nts effect platelet aggregation is not known but has generally be n ascribed to the release of ADP from the platelets, based on the inhibitory effects of apyrase, with little contribution from the activation of the coagulation system.

The relative contribution of these 2 systems in tumor cell-induced platelet aggregation may be evaluated by using specific enzymes. Apyrase removes ADP from solution by converting it to AMP. Thus, inhibition of aggregation by apyrase implies the involvement of ADP. A similar conclusion can be drawn if inhibition by a mixture of phosphoenolpyruvate-pyruvate kinase is observed. This mixture converts ADP to ATP and, because of the speed of the reaction in comparison to apyrase, can inhibit processes in which considerable amounts of ADP are being produced. Hirudin is a polypeptide which is a specific inhibitor of thrombin, and hence, inhibition by hirudin is taken to indicate the inhibition of procoagulant-d pendent reactions.

Most of the previous work has utilized animal tumor cell lin s, mainly of rat or mouse origin, and human or rabbit

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platelets. We have recently begun to reexamine the interaction of platelets and tumor cells (3), their attachment as mixed thrombi at the vessel wall (16), and idiosyncratic aggregation responses to tumor cells by platelets from different donors (2) utilizing homologous systems of well-characterized cell lines derived from human tumors and heparinized human PRP.[4] Two different mechanisms of platelet aggregation have been identified in these studies. With the Hut20 line, derived from an anaplastic murine tumor, the onset of aggregation appeared to be dependent on ADP derived from the tumor cells but not from the platelets, since it occurred prior to the onset of the platelet release reaction. Aggregation occurred using platelets from donors who had ingested aspirin but was completely inhibited in the presence of apyrase and was unaffected by hirudin. On the other hand, aggregation by the U87MG (human glioblastoma) line appeared to be due to a procoagulant activity released from the tumor cells since it was completely inhibited by hirudin but was unaffected by apyrase. Furthermore, phospholipase D, which cleaves phosphatidylcholine to choline and phosphatidic acid, inhibited platelet aggregation induced by Hut20 tumor cells while aggregation induced by U87MG cells was unaffected by the enzyme (3).

In the present studies with a further 6 human tumor cell lines, we have found that thrombin-mediated systems in the 2 cell lines can be differentiated on the basis of their sensitivities to phospholipase D. ADP-dependent processes appear to be the major mechanism modulating tumor cell-induced platelet aggregation in the homologous human systems examined thus far.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade. Heparin sodium was obtained from Fisher Scientific Co., Pittsburgh, Pa., while the following products were obtained from Sigma Chemical Co., St. Louis, Mo.: hirudin, Grade IV, from leeches (activity, 1000 units/mg protein); phospholipase A_2 from bee venom (1500 units/mg); phospholipase C type III from *Bacillus cereus* (80 units/mg); phospholipase D type I from cabbage (100 units/mg); phosphoenolpyruvate trisodium salt; pyruvate kinase type II from rabbit muscle; bovine tendon collagen; ADP (Grade I); L-β-lysophosphatidylcholine (L-β-lysolecithin; type III, from bovine liver). Apyrase, Grade 1, from potato (ADP activity, 500 milliunits/mg) was obtained from Sigma and was shown to be free of detectable protease activity using agar plates containing either albumin or casein (5).

Tumor Cells and Cell Cultures. The HT29 (adenocarcinoma), SKNMC (neuroblastoma), SKBR3 (adenocarcinoma), and HT144 (melanoma) lines were provided by Dr. Jørgen Fogh, Sloan-Kettering Institute, Rye, N. Y., while the Hut23 (adenocarcinoma) and Hut28 (mesothelioma) lines were provided by Dr. Adi Gazdar, Veterans Administration Hospital, Washington, D. C.

Cells were grown to confluency in 150-ml polycarbonate flasks (Falcon Plastics, Oxnard, Calif.) in a tissue culture incubator in an atmosphere of 95% O_2-5% CO_2. Hut23 and Hut28 lines were maintained in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal calf serum. The SKNMC line was maintained in minimum essential medium with Earle's salts supplemented with 1% nonessential amino acids and 15% fetal calf serum. SKBR3, HT144, and HT29 cell lines were maintained in McCoy's Medium 5A supplemented with 15% of fetal calf serum. Fifty units penicillin per ml and 50 mg streptomycin per ml were included in all media used.

Cells were harvested without exposure to proteases by decanting the culture medium, washing the monolayers twice with HBSS, and then treating them for 5 min with HBSS free of Ca^{2+} and Mg^{2+} and containing 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. The cell suspension was centrifuged at 800 × *g* for 10 min, the supernatant solution was removed, and the cell pellets were washed twice with a solution of HBSS free of Ca^{2+} and Mg^{2+} but containing 0.2% bovine serum albumin and were finally resuspended in the same solution but without apyrase. Cells were counted in a hemocytometer, and viability was determined by exclusion of trypan blue. The range of viable cells was 90 to 97%. None of the cell lines showed contamination with fibroblasts, and the effects observed were not affected by treatment of the cultures with collagenase.

Aggregometry. Blood from healthy laboratory staff, who had not taken aspirin or related drugs in the previous 7 days, was collected using heparin (5 units/ml) as anticoagulant. Centrifugation of whole blood was performed at 150 × *g* for 10 min in plastic tubes and PRP was removed by aspiration. Platelet counts were determined and were in the normal range (250,000 to 350,000/cu mm) for all samples examined. Platelet aggregation was measured in an aggregometer (Chronolog Corp., Broomall, Pa.) at 37° with constant stirring at 1000 rpm using 450 μl of PRP and 50 μl of the tumor cell suspension.

Modification of Aggregation Response. To study the role of the development of procoagulant activity, hirudin (100 units/ml) was incubated with PRP for 30 min at room temperature prior to the addition of tumor cells to the system. For studying the effects of apyrase, the enzyme was added at a level of 125 μg/ml to PRP immediately prior to the addition of tumor cells. For studies with the phospholipases, the enzymes were added to PRP in the aggregometer cuvet immediately prior to the aggregating dose of tumor cells. The phospholipases were all used at a concentration of 10 units/ml (A_2, 7 μg/ml; C, 125 μg/ml; D, 100 μg/ml). None of these agents themselves caused aggregation on prolonged incubation with heparinized PRP in the absence of tumor cells.

RESULTS

Aggregation by Tumor Cells. Each of the cell lines examined produced different aggregation patterns and required different amounts of tumor cells to effect aggregation. Aggregation was dependent only on cell number and not on the degree of confluency when cells were harvested at earlier points in the growth cycle. The HT29 line aggregated platelets at a final concentration in the cuvet of 10^6 cells/ml. The Hut28, HT144, SKBR3, and SKNMC lines required 5×10^6 cells/ml while the Hut23 line did not cause platelet aggregation at tumor cell concentrations as high as 10^7/ml. Differences were also observed between the different cell lines in the effects of apyrase, hirudin, and phospholipase D on the course of aggregation. These results are described below for each of the cell lines examined.

SKBR3. The aggregation profile of platelets exposed to SKBR3 cells consisted of a brief phase of reversible aggregation followed immediately by a larger irreversible phase (Chart 1*A*, *Curve a*). This profile is similar to that observed previously with the Hut20 line (3). Aggregation by this cell line was completely blocked by apyrase (250 μg/ml) (Chart 1*A*, *Curve b*). The second wave of aggregation, but not the first, was inhibited by phospholipase D (Chart 1*A*, *Curve c*). Hirudin (100 units/ml) had no effect on the aggregation profile (Chart 1*A*, *Curve d*).

SKNMC. A similar pattern of biphasic aggregation was observed with the SKNMC cell line although in this case there was a prolonged delay between the reversible wave and the

[4] The abbreviations used are: PRP, platelet-rich plasma; HBSS, Hanks' balanced salt solution.

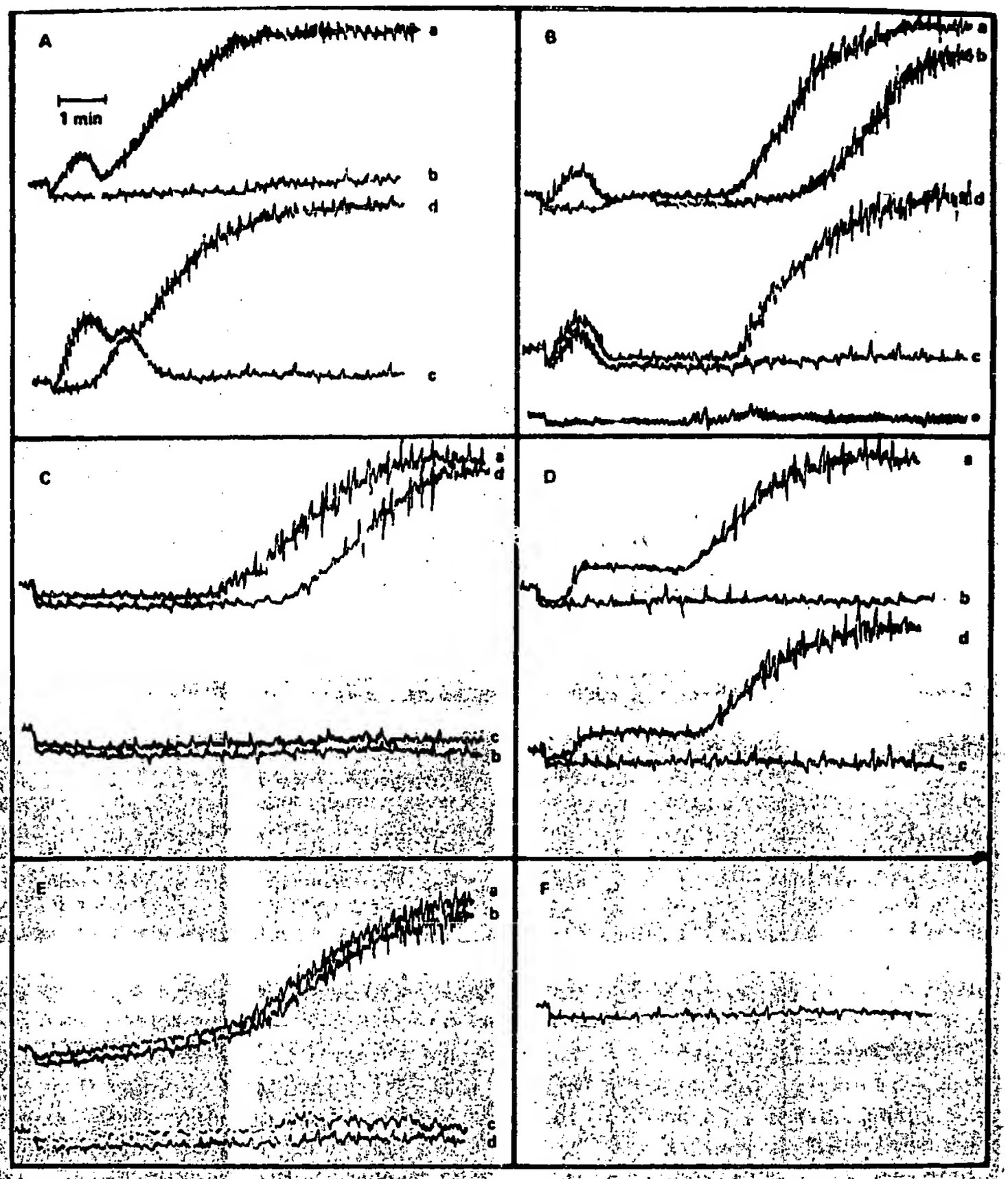

Chart 1. Aggregation of platelets in human heparinized PRP by different human tumor cell lines and modification of aggregation responses. In each *panel*, *Curve a* is aggregation induced by tumor cells alone at the concentrations described below, *Curve b* is the aggregation produced in the presence of apyrase (120 μg/ml), *Curve c* is the aggregation produced in the presence of phospholipase D (10 units/ml), and *Curve d* is the aggregation produced in the presence of hirudin (100 units/ml). For the SKNMC line, *Curve e* shows the effect of phosphoenolpyruvate-pyruvate kinase. Apyrase and phospholipase D were added to the PRP in the aggregometer cuvet immediately prior to the addition of the aggregating dose of tumor cells, while hirudin was incubated with the PRP for 30 min prior to the addition of tumor cells. *A*, SKBR3, 5×10^6 cells/ml; *B*, SKNMC, 5×10^6; *C*, HT144, 5×10^6; *D*, HT29, 10^6; *E*, Hut28, 5×10^6; *F*, Hut23, 10^7.

nset of the second wave of aggregation (Chart 1*B*, *Curve a*). Apyrase caused a delay in the onset of the first wave of aggregation and a reduction in its amplitude as well as an increase in the lag time to the second wave of irreversible aggregation (Chart 1*B*, *Curve b*). In order to clarify the possible involvement of ADP, studies were also carried out with phosphoenolpyruvate-pyruvate kinase which destroys ADP more effectively than apyrase. This combination resulted in complete inhibition of both waves of the aggregation induced by SKNMC cells (Chart 1*B*, *Curve e*). Incubation with phospholipase D resulted in the inhibition of the second wave of aggregation, but the first wave was unaffected (Chart 1*B*, *Curve c*), while hirudin had no effect on the course of platelet aggregation induced by the SKNMC line (Chart 1*B*, *Curve d*).

HT144. The HT144 line caused monophasic irreversible aggregation with a prolonged lag time (Chart 1*C*, *Curve a*). In some experiments, there appeared to be a very small first wave during this lag period, but this effect was not consistently observed. Both apyrase (Chart 1*C*, *Curve b*) and phospholipase D (Chart 1*C*, *Curve c*) completely blocked aggregation with HT144, while hirudin was without effect other than causing a slight prolongation of the lag phase preceding irreversible aggregation (Chart 1*C*, *Curve d*).

HT29. The HT29 line also showed biphasic aggregation but with no apparent dissociation between the first and second waves and with a brief lag phase preceding aggregation (Chart 1*D*, *Curve a*). Aggregation by this cell line was completely blocked in the presence of apyrase (Chart 1*D*, *Curve b*) or phospholipase D (Chart 1*D*, *Curve c*). On the other hand, neither the lag phase preceding aggregation nor the biphasic aggregation curve was affected by the presence of hirudin (Chart 1*D*, *Curve d*).

Hut28. Hut28 cells caused monophasic aggregation but with a slowly increasing base line during the prolonged lag phase preceding aggregation (Chart 1*E*, *Curve a*). Apyrase had no effect on aggregation (Chart 1*E*, *Curve b*), but it was completely inhibited by phospholipase D (Chart 1*E*, *Curve c*) and by hirudin (Chart 1*E*, *Curve d*).

Hut23. The Hut23 cell line produced no significant aggregation at tumor cell concentrations as high as 10^7/ml (Chart 1*F*, *Curve a*).

Effects of Phospholipases and Lysolecithin. As noted

above, phosphclipase D showed differential effects on the aggregation produced by the various cell lin s. Phospholipase C (10 units/ml) had no ffect on platelet aggregation produc d by any of the cell lines xamined, while phosph lipase A_2, add d at th same time as the tumor cells, compl t ly blocked aggregation by all of the human tumor cell lines. Lysolecithin, the product of the action of phospholipase A_2 on phosphatidylcholine, at a concentration of 50 μg/ml, also caused compl te inhibition of aggregation with all of the tumor cell lines examined (data not shown).

DISCUSSION

In previous studies, we have examined platelet aggregation induced by 2 tumor cell lines, Hut20 from an undifferentiated murine tumor and U87MG derived from a human glioblastoma (3). Two different mechanisms of induction of platelet aggregation were apparent. Platelet aggregation induced by the Hut20 line appeared to be primarily dependent on ADP released from the tumor cells themselves, while aggregation induced by the U87MG line was dependent on a procoagulant activity elaborated by the tumor cell surface.

The present examination of a further 6 human tumor cell lines shows that a third mechanism of platelet aggregation by cultured human tumor cells can be recognized based on aggregation responses and the inhibitory effects of apyrase, hirudin, and phospholipase D. The results are summarized in Table 1 together with the results for the Hut20, A549, and U87MG lines examined previously (3, 16). Some groupings among the various lines appear to be possible. In all cases, aggregation occurred only with heparinized PRP and not when citrate was used as anticoagulant.

SKBR3 induces biphasic platelet aggregation that is inhibited by apyrase but not by hirudin, and phospholipase D eliminates the second wave of aggregation. This pattern is similar to that observed previously with the Hut20 line. In this case, secretion of ADP from the tumor cells initiates the first wave of aggregation, which leads to platelet activation and to a second wave of aggregation. This second wave is associated with platelet secretion which must be independent of thrombin production since it is not blocked by hirudin. SKNMC, HT144, and HT29 also probably belong in this class. With SKNMC, the lag phase was prolonged with apyrase and aggregation was completely inhibited by phosphoenolpyruvate-pyruvate kinase. With HT144 and Hut29, there was no clearly marked reversible first wave, but platelet aggregation was completely inhibited by apyrase. No effect was observed with hirudin for any of th se 3 lines.

Aggregation induced by the Hut28 lines shows a rising base lin prior to the onset of aggregation and is unaffected by apyrase but is completely blocked by phospholipase D and hirudin. The effects of apyrase and hirudin are identical to thos se n with the U87MG line xamined previously (3), suggesting that the aggregation effects of these 2 lines involves activation of the coagulation system. However, platelet aggregation by these 2 lines may be differentiated since only with the Hut28 line is aggregation inhibited by phospholipase D.

The various phospholipases have been of value in characterizing the platelet-aggregating effects of the tumor cell lines. The effects of phospholipase D on tumor cell-induced platelet aggregation have not been investigated previously, but this enzyme was able to differentiate the platelet-aggregating effects of Hut28 from those of U87MG although activation of th coagulation system appeared to be involved in each case. For those lines showing a clearly marked phase of reversible aggregation (SKBR3, SKNMC, and HT29), it may be noted that phospholipase D inhibited the second, major wave. For HT29 and Hut28, where a reversible first wave was not detectable, the aggregation response was also inhibited by phospholipase D. Little is known about the effects of phospholipase D on membranes, but the enzyme can alter calcium translocation in sarcoplasmic reticulum (6), and this may explain its differential effects on the first and second waves of tumor cell-induced platelet aggregation.

Phospholipase A_2 has been reported to completely inhibit platelet aggregation induced by 2 mouse tumor cell lines (13, 17) as well as by the 5 human tumor lines which caused aggregation in the present study. We have also found that tumor cell-induced platelet aggregation is inhibited by lysolecithin, the product of the action of phospholipase A_2 on phosphatidylcholine. Lysolecithin also inhibits platelet aggregation induced by ADP, epinephrine, collagen, and thrombin (15). It is known to inhibit prostaglandin synthesis (19), and this may be the basis for its antiaggregating effects, although it can also affect membrane fluidity (21) and the levels of nucleotid cyclases (1, 20).

In addition to the usual aggregating agents, platelet aggregation can be induced by the platelet-aggregating factor elaborated by IgE-sensitized basophils. Platelet-aggregating fact r is lipidic in nature and is destroyed by phospholipases A_2, C, and D (4). Since phospholipase C had no effect on platel t aggregation induced by any of the human tumor cell lines

Table 1

Summary of cell lines examined for platelet-aggregating ability, aggregation profiles, and inhibitor effects

Cell line	Origin	Cell concentration	Aggregation	Inhibition by: Apyrase	Inhibition by: Hirudin	Inhibition by: Phospholipase D
SKBR3	Adenocarcinoma of breast	5×10^6	Biphasic	Inhibition	No inhibition	2nd wave only
Hut20[a]	Anaplastic murine tumor	10^5	Biphasic	Inhibition	No inhibition	2nd wave only
SKNMC	Neuroblastoma	5×10^6	Biphasic	Inhibition[b]	No inhibition	2nd wave only
HT144	Melanoma	5×10^6	Monophasic	Inhibition	No inhibition	Inhibition
HT29	Adenocarcinoma of colon	10^6	Biphasic	Inhibition	No inhibition	Inhibition
Hut28	Mesothelioma	5×10^6	Monophasic	No inhibition	Inhibition	Inhibition
U87MG[a]	Glioblastoma	10^5	Monophasic	No inhibition	Inhibition	No inhibition
Hut23	Poorly differentiated adenocarcinoma	10^7	None			
A549[a]	Small-cell lung carcinoma	10^7	None			

[a] Lines examined previously (3, 17) are included for comparison.
[b] Partial inhibition with apyrase, complete inhibition with phosphoenolpyruvate-pyruvate kinase.

examined, it is unlikely that platelet-aggregating factor-like material is involved in this reaction.

In summary, our results suggest that there are 2 major mechanisms by which cultured human tumor cells initiate platelet aggregation. The major mechanism, in 4 of the 6 aggregating human lines so far examined, appears to involve the secretion of ADP from the tumor cells resulting in platelet stimulation and then irreversible aggregation. This secretion may reflect damage to the tumor cells during harvesting, but the secretion of ADP from tumor cells in this group could be of physiological significance, and since aggregation does not occur in the presence of citrate, it differs from the usual mechanisms of ADP-induced platelet aggregation. The second mechanism involves the initial activation of the coagulation system and the generation of thrombin as the mediator of aggregation. Within this second group, 2 subgroups can be identified based on whether or not aggregation can be inhibited by phospholipase D. However, the different patterns of aggregation and inhibition within different groups of tumor cells, considerations of the relative importance of ADP and the coagulation system, and the observation of different patterns of inhibition with phospholipase D all suggest that no single mechanism will explain the nature of the interaction between platelets and tumor cells under all circumstances. These preliminary studies also suggest that significant differences exist between mechanisms of tumor cell-induced platelet aggregation seen previously in heterologous animal systems (8, 10, 12–14, 17, 18) and those seen in the homologous human systems studied here.

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L10 ANSWER 9 OF 9 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 79104252 MEDLINE
DOCUMENT NUMBER: 79104252 PubMed ID: 216440
TITLE: In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency.
AUTHOR: Lee C H; Evans S P; Rozenberg M C; Bagnara A S; Ziegler J B; Van der Weyden M B
SOURCE: BLOOD, (1979 Mar) 53 (3) 465-71.
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DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
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FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 197904
ENTRY DATE: Entered STN: 19900315
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AB The platelets of an infant with severe combined immune deficiency and adenosine deaminase deficiency showed markedly diminished responses to ADP-induced aggregation in vitro. This abnormality was corrected by the addition of purified adenosine deaminase in vitro. **Exogenous adenosine** added to platelet-rich plasma caused markedly prolonged inhibition of ADP-induced aggregation. This was shown by isotopic studies to be due to slow clearance of adenosine and hence persistence of this nucleoside. Direct assay for adenosine deaminiase in plasma and platelet lysates of the patient confirmed the very low activity of this enzyme. Raised cAMP levels were demonstrated in his platelets. The deranged adenosine metabolism and raised cAMP in the platelets of this child with severe combined immunodeficiency may explain the altered response to ADP. Despite the in vitro **platelet aggregation** abnormality, the patient had no clinical evidence of impaired hemostasis.

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L9 ANSWER 49 OF 107 CAPLUS COPYRIGHT 2003 ACS
ACCESSION NUMBER: 1990:545076 CAPLUS
DOCUMENT NUMBER: 113:145076
TITLE: Effect of **adenosine** and inosine administration on platelet function in rabbits
AUTHOR(S): Singh, J.; Sudhir, S.; Gupta, L. C.; Garg, K. N.
CORPORATE SOURCE: Dep. Pharmacol., Med. Coll. Hosp., Rohtak, 124 001, India
SOURCE: Indian Journal of Physiology and Pharmacology (1990), 34(1), 63-4
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LANGUAGE: English

AB Rabbits receiving adenosine, inosine, and aspirin exhibited a marked increase in platelet aggregation times. All the 3 agents also decreased platelet adhesiveness. These agents do not differ much in potency, since effects were comparable with similar doses. The antiaggregation effect of adenosine may be due to its action on extracellular membrane receptors or due to stabilization of the intracellular dense granules, there-by preventing the "release I reaction". The antiaggregation effect of inosine may be due to similar mechanisms.

TI Effect of **adenosine** and inosine administration on platelet function in rabbits

L9 ANSWER 49 OF 107 CAPLUS COPYRIGHT 2003 ACS
ACCESSION NUMBER: 1990:545076 CAPLUS
DOCUMENT NUMBER: 113:145076
TITLE: Effect of **adenosine** and inosine administration on platelet function in rabbits
AUTHOR(S): Singh, J.; Sudhir, S.; Gupta, L. C.; Garg, K. N.
CORPORATE SOURCE: Dep. Pharmacol., Med. Coll. Hosp., Rohtak, 124 001, India
SOURCE: Indian Journal of Physiology and Pharmacology (1990), 34(1), 63-4
CODEN: IJPPAZ; ISSN: 0019-5499
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Rabbits receiving adenosine, inosine, and aspirin exhibited a marked increase in platelet aggregation times. All the 3 agents also decreased platelet adhesiveness. These agents do not differ much in potency, since effects were comparable with similar doses. The antiaggregation effect of adenosine may be due to its action on extracellular membrane receptors or due to stabilization of the intracellular dense granules, there-by preventing the "release I reaction". The antiaggregation effect of inosine may be due to similar mechanisms.

TI Effect of **adenosine** and inosine administration on platelet function in rabbits

Gabel, Gailene

To: STIC-ILL
Subject: 09/853,524

Please provide a copy of the following literature ASAP:

1) KUTSUNA et al., Identification and determination of platelet aggregation inhibitor from safflower (Carthamus tinctorius Linne). JOURNAL OF THE PHARMACEUTICAL SOCIETY OF JAPAN, (1988 Nov) 108 (11) 1101-3.

Thanks a bunch,
Gailene Gabel
7B15
305-0807

WEST Search History

DATE: Monday, January 06, 2003

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
L15	5780590.pn. and adenosine	1	L15
L14	5780303.pn. and adenosine	1	L14
L13	5480303.pn. and adenosine	0	L13
L12	5773411.pn. and adenosine	1	L12
L11	57734115780303.pn. and adenosine	0	L11
L10	5780303.pn. and adenosine	1	L10
L9	5731324.pn. and adenosine	1	L9
L8	5681823.pn. and adenosine	1	L8
L7	5672585.pn. and adenosine	1	L7
L6	5668159.pn. and adenosine	1	L6
L5	5635477.pn. and adenosine	1	L5
L4	5618843.pn. and adenosine	1	L4
L3	5612311.pn. and adenosine	1	L3
L2	5053393.pn. and adenosine	1	L2
L1	4879313.pn. and adenosine	1	L1

END OF SEARCH HISTORY

WEST Search History

DATE: Monday, January 06, 2003

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
L5	L1 and glycoprotein$1	0	L5
L4	L1 and (platelet aggregat$4)	6	L4
L3	L1 and (thrombo$8)	9	L3
L2	L1 and (platelet aggregat?)	0	L2
L1	exogenous adenosine	34	L1

END OF SEARCH HISTORY

(FILE 'HOME' ENTERED AT 15:57:14 ON 06 JAN 2003)

FILE 'MEDLINE, EMBASE, SCISEARCH, BIOSIS, USPATFULL' ENTERED AT 15:57:41 ON 06 JAN 2003

L1	7197	S ADENOSINE (6P) (PLATELET AGGREGAT?)
L2	1401	S ADENOSINE (6P) (THROMBOSIS)
L3	266	S ADENOSINE (6P) (THROMBOEMBOLIC)
L4	86	S L1 AND L2 AND L3
L5	68	DUP REM L4 (18 DUPLICATES REMOVED)
L6	1537	S EXOGENOUS ADENOSINE
L7	16	S L6 (6P) (PLATELET AGGREGAT?)
L8	5	S L6 (6P) (THROMBOSIS)
L9	0	S L6 (6P) (THROMBOEMBOLIC)
L10	9	DUP REM L7 (7 DUPLICATES REMOVED)
L11	2	DUP REM L8 (3 DUPLICATES REMOVED)
L12	18	S L6 (6P) (THROMBO? OR ANTITHROMBO?)
L13	10	DUP REM L12 (8 DUPLICATES REMOVED)
L14	917	S EXOGENOUS ADENOSINE/AB
L15	287	S EXOGENOUS ADENOSINE/TI
L16	38	S L15 AND L14
L17	17	DUP REM L16 (21 DUPLICATES REMOVED)
L18	17	S L6 AND L1
L19	7	S L6 AND L2
L20	0	S L6 AND L3
L21	10	DUP REM L18 (7 DUPLICATES REMOVED)
L22	4	DUP REM L19 (3 DUPLICATES REMOVED)
		E CHANG/AU
L23	46	S E3
L24	0	S L23 AND ADENOSINE
		E HSU/AU
L25	4	S E3
L26	0	S L25 AND ADENOSINE

=>

(FILE 'HOME' ENTERED AT 17:14:12 ON 06 JAN 2003)

FILE 'MEDLINE, EMBASE, SCISEARCH, BIOSIS, USPATFULL' ENTERED AT 17:14:30
ON 06 JAN 2003

L1	1900	S (ADENOSINE (2A) EXOGENOUS)
L2	0	S L1 (6P) (GLYCOPROTEIN? OR GPIIBIIIA)
L3	11	S L1 AND (GLYCOPROTEIN? OR GPIIBIIIA)
L4	8	DUP REM L3 (3 DUPLICATES REMOVED)

Gabel, Gailene

To: STIC-ILL
Subject: 09/853,524

Please provide a copy of the following literature:

1) Rubin et al., New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. Journal of Vascular Surgery, (1992) 15/4 (683-692).

2) Armstrong et al., Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection), THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.

3) ELY et al., PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA, CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904.

4) Bastida et al., Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin, and phospholipase. Cancer Research, (1982) 42/11 (4348-4352).

5) Lee et al., In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency. BLOOD, (1979 Mar) 53 (3) 465-71.

6) Cattaneo et al., Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. BLOOD, (1990 Mar 1) 75 (5) 1081-6.

7) Wang et al., Exogenous adenosine application inhibits thrombus formation in stenosed canine coronary artery and partially protects against renewal of thrombus formation by epinephrine. FASEB Journal, (1995) Vol. 9, No. 3, pp. A322.

8)

ACCESSION NUMBER: 2002:157636 USPATFULL
TITLE: Adenosine as antithrombotic
INVENTOR(S): Chang, Su-Chen, Taichung, TAIWAN, PROVINCE OF CHINA
Hsu, Li-Wei, Taichung, TAIWAN, PROVINCE OF CHINA

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 2002082241	A1	20020627
APPLICATION INFO.:	US 2001-853524	A1	20010510 (9)

RELATED APPLN. INFO.: Continuation-in-part of Ser. No. US 2000-708306, filed on 7 Nov 2000, PENDING
DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: Kate H. Murashige, Morrison & Foerster LLP, Suite 500, 3811 Valley Centre Drive, San Diego, CA, 92130-2332
NUMBER OF CLAIMS: 18
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 12 Drawing Page(s)
LINE COUNT: 590
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention discloses a specific binding of **adenosine** to a platelet membrane receptor protein gpIIb/IIIa, and relates to the novel use of **adenosine** for inhibiting **platelet aggregation** and **thrombosis**. The present invention discloses that **adenosine** is useful as an antithrombotic.

AB The present invention discloses a specific binding of **adenosine** to a platelet membrane receptor protein gpIIb/IIIa, and relates to the novel use of **adenosine** for inhibiting **platelet aggregation** and **thrombosis**. The present invention discloses that **adenosine** is useful as an antithrombotic.

SUMM [0002] The present invention relates to the novel use of **adenosine** as an antagonist against a platelet membrane receptor protein gpIIb/IIIa for inhibiting **platelet aggregation** and **thrombosis**.

SUMM [0003] Most of the **thromboembolic** disorders, including atherosclerosis and arteriosclerosis, acute myocardial infarction, angina, transient ischemic attacks and strokes, peripheral vascular diseases, arterial **thrombosis**, preeclampsia, embolism and carotid endarterectomy, are related to the formation of blood clot or thrombus in blood vessel. **Platelet aggregation** plays an important role in the thrombus formation. It was found that **platelet aggregation** is dependent upon the binding of fibrinogen and other serum proteins to a platelet membrane receptor protein gpIIb/IIIa located on. . . as thrombin, the gpIIb/IIIa binding site becomes available to fibrinogen and other serum proteins for binding, thereby resulting in the **platelet aggregation** and thrombus formation. Thus, inhibition of the binding of fibrinogen and other serum proteins to gpIIb/IIIa is a requisite for. . .

SUMM [0004] The development of a gpIIb/IIIa antagonist represents a promising approach to inhibiting **platelet aggregation** and **thrombosis**. A gpIIb/IIIa-specific antiplatelet agent which can inhibit the activation and aggregation of platelets in response to any agonist is therefore. . .

SUMM [0005] Various products such as aspirin against arachidonic acid, ticlopidine against **adenosine** diphosphate (ADP), hirudin against thrombin, and thromboxane A.sub.2 synthase inhibitors or receptor antagonists against thromboxane A.sub.2 synthase, are available now for preventing **platelet aggregation** and thrombus formation. However, these products do not specifically inhibit the binding of fibrinogen and other serum proteins to gpIIb/IIIa. . .

SUMM . . . having a nucleus formed of two fused six membered rings, which were useful as gpIIb/IIIa antagonists for the inhibition of **platelet aggregation**.

SUMM . . . 5,672,585 and 5,612,311 disclosed certain cyclic peptides having a high affinity for gpIIb/IIIa, which were useful in the inhibition of **platelet aggregation** and thus in the treatment of **thrombosis**.

SUMM . . . disclosed certain cyclic compounds linked by a heterocyclic ring system, which were useful as gpIIb/IIIa antagonists for the treatment of **thrombosis**.

SUMM [0009] U.S. Pat. Nos. 5,053,393 disclosed N-[8-[(aminoiminomethyl)amino]-1-oxooctyl]-N-L-.alpha.-aspartyl-L-phenylalanine as a potent compound for the inhibition of **platelet aggregation**.

SUMM [0010] U.S. Pat. Nos. 4,879,313 disclosed certain peptide mimetic compounds, which were useful in the inhibition of **platelet aggregation** and in the treatment of **thrombosis**.

SUMM . . . tinctorius L for active ingredients that can specifically bind to gpIIb/IIIA, and surprisingly found that a small compound, identified as **adenosine**, can strongly bind to gpIIb/IIIa and thus can inhibit **platelet aggregation** and **thrombosis**, especially in view of the fact that ADP was well known in the art as an endogenous agonist for activating platelets. Accordingly, the inventors of the present invention found that **adenosine** is a potent antithrombotic.

SUMM [0015] **Adenosine**, a compound well known in the art, was used as an antiarrhythmic, and its derivatives were used as anti-tumor agents. The therapeutic use of **adenosine** for specifically binding to gpIIb/IIIa and treating **thromboembolic** disorders was never disclosed or suggested in any prior art reference.

SUMM [0016] Based on the above, the inventors of the present invention found a new therapeutic use or indication of **adenosine** in the inhibition of **platelet aggregation** and thrombus formation.

SUMM [0018] The present invention also relates to the novel use of **adenosine** as an antagonist against gpIb/IIIa for inhibiting **platelet aggregation** and **thrombosis**.

SUMM [0019] One aspect of the present invention relates to a method for inhibiting **platelet aggregation** and **thrombosis** by administering to a mammal an effective amount of **adenosine**.

SUMM [0020] Another aspect of the present invention relates to a method for preventing and treating **thromboembolic** disorders including atherosclerosis and arteriosclerosis, acute myocardial infarction, angina, transient ischemic attacks and strokes, peripheral vascular diseases, arterial **thrombosis**, preeclampsia, embolism and carotid endarterectomy by administering to a mammal an effective amount of **adenosine**.

SUMM . . . relates to an in vitro method for detecting the presence of gpIIb/IIIa in a suspected sample by contacting gpIIb/IIIa with **adenosine**.

SUMM [0022] Another further aspect of the present invention relates to a pharmaceutical composition for inhibiting **platelet aggregation** and **thrombosis** in a mammal, comprising an effective amount of **adenosine** and a pharmaceutically acceptable carrier or diluent.

SUMM [0023] Another further aspect of the present invention relates to a pharmaceutical composition for preventing and treating **thromboembolic** disorders including atherosclerosis and arteriosclerosis, acute myocardial infarction, angina, transient ischemic attacks and strokes, peripheral vascular diseases, arterial **thrombosis**, preeclampsia, embolism and carotid endarterectomy in a mammal, comprising an effective amount of **adenosine** and a pharmaceutically acceptable carrier or diluent.

SUMM [0024] Another further aspect of the present invention relates to a kit for inhibiting **platelet aggregation** and **thrombosis** in a mammal, comprising a first container containing **adenosine** and a second container containing a pharmaceutically

acceptable carrier or diluent.

L5 ANSWER 30 OF 68 USPATFULL
ACCESSION NUMBER: 96:92076 USPATFULL
TITLE: Aromatic compounds containing basic and acidic termini useful as fibrinogen receptor antagonists
INVENTOR(S): DeGrado, William F., Moylan, PA, United States
Xue, Chu-Biao, Hockessin, DE, United States
PATENT ASSIGNEE(S): The Dupont Merck Pharmaceutical Company, Wilmington, DE, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5563158		19961008	
APPLICATION INFO.:	US 1994-343159		19941122	(8)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1993-174552, filed on 28 Dec 1993, now abandoned			
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Davis, Zinna Northington			
NUMBER OF CLAIMS:	22			
EXEMPLARY CLAIM:	1			
LINE COUNT:	4191			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB This invention relates to novel compounds containing basic and acidic termini, pharmaceutical compositions containing such compounds, processes for preparing such compounds, and to methods of using these compounds, alone or in combination with other therapeutic agents, for the inhibition of platelet aggregation, as thrombolytics, and/or for the treatment of thromboembolic disorders.

SUMM . . . compounds, and to methods of using these compounds, alone or in combination with other therapeutic agents, for the inhibition of **platelet aggregation**, as thrombolytics, and/or for the treatment of **thromboembolic** disorders.

SUMM . . . the injured area by a phenomenon called platelet adhesion. Activated platelets also bind to each other in a process called **platelet aggregation** to form a platelet plug. This platelet plug can stop bleeding quickly, but it must be reinforced by fibrin for. . .

SUMM **Thrombosis** may be regarded as the pathological condition wherein improper activity of the hemostatic mechanism results in intravascular thrombus formation. Activation of platelets and the resulting **platelet aggregation** and platelet factor secretion has been associated with different pathophysiological conditions including cardiovascular and cerebrovascular **thromboembolic** disorders, for example, the **thromboembolic** disorders associated with unstable angina, myocardial infarction, transient ischemic attack, stroke, atherosclerosis, and diabetes. The contribution of platelets to these. . .

SUMM Platelets are known to play an essential role in the maintenance of hemostasis and in the pathogenesis of arterial **thrombosis**. Platelet activation has been shown to be enhanced during coronary thrombolysis. This can lead to delayed reperfusion and reocclusion. Clinical. . .

SUMM . . . of platelets serves to further focus clot formation by concentrating activated clotting factors in one site. Several endogenous agonists, including **adenosine** diphosphate (ADP), serotonin, arachidonic acid, thrombin, and collagen, have been identified. Because of the involvement of several endogenous agonists in. . .

SUMM . . . known agonists has been identified, namely the platelet glycoprotein IIb/IIIa complex (GPIIb/IIIa or IIb/IIIa), which is the membrane protein mediating **platelet aggregation**. A recent review of GPIIb/IIIa is provided by Phillips et al. (1991) Cell 65: 359-362. The development of a GPIIb/IIIa. . .

SUMM . . . IIb/IIIa receptor, thereby preventing fibrinogen from binding at its platelet receptor site, leading to efficacy in the prevention of blood **platelet aggregation** and subsequent clotting disorders.

=>

L5 ANSWER 41 OF 68 EMBASE COPYRIGHT 2003 ELSEVIER SCI. B.V.DUPLICATE 6
ACCESSION NUMBER: 92177694 EMBASE
DOCUMENT NUMBER: 1992177694
TITLE: New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts.
AUTHOR: Rubin B.G.; McGraw D.J.; Sicard G.A.; Santoro S.A.
CORPORATE SOURCE: Department of Surgery, 216 South Kingshighway,St. Louis, MO 63110, United States
SOURCE: Journal of Vascular Surgery, (1992) 15/4 (683-692).
ISSN: 0741-5214 CODEN: JVSUES
COUNTRY: United States
DOCUMENT TYPE: Journal; Conference Article
FILE SEGMENT: 030 Pharmacology
037 Drug Literature Index
LANGUAGE: English
SUMMARY LANGUAGE: English

AB Platelet adhesion and aggregation are mediated by fibrinogen via the receptor glycoprotein IIb/IIIa, which recognizes the arginine-glycine-aspartic (RGD) amino-acid sequence. We investigated the ability of 8-guanidino-octanoyl-Asp-Phe (SC-49992), an intravenously infused, stable RGD analogue, to inhibit human platelet function in vitro and to reduce in vivo canine platelet deposition on prosthetic grafts. Human **platelet aggregation** induced by 10 .mu.mol/L **adenosine** diphosphate was inhibited in a concentration dependent manner with an ED50 of 1 .mu.g/ml of SC-49992. **Adenosine** diphosphate-induced secretion, which is dependent on fibrinogen occupancy of the glycoprotein IIb/IIIa receptor, was reduced in a concentration dependent manner, also with an ED50 of 1 .mu.g/ml. Thrombin-induced secretion, which is independent of fibrinogen binding, was unaffected. Activation-dependent platelet adhesion to fibrinogen substrates was reduced in a concentration- dependent manner by SC-49992. Platelet adhesion to fibronectin substrates was also reduced by the analogue, but to a lesser extent. SC-49992 effectively eluted glycoprotein IIb/IIIa bound to RGD derivatized sepharose. Eight **thrombosis**-prone dogs had polytetrafluoroethylene femoral artery grafts placed. Dogs received the RGD analogue or a normal saline infusion during their first graft procedure. One week later a second contralateral femoral graft with infusion of the other agent was performed. Aggregometry during RGD analogue infusion demonstrated inhibition of induced aggregation, whereas normal saline infusion had no effect. As measured by the adherence of platelets labeled with indium III 8-guanidino-octanoyl-Asp-Phe reduced platelet deposition on vascular grafts by more than 90% (p = 0.0006, log transformed data, paired t test). Histologic examination demonstrated marked reduction or complete elimination of platelet thrombus on the luminal surface of the grafts under drug-treated conditions. Previous attempts to block **platelet aggregation** have been of limited success. 8-guanidino-octanoyl-Asp- Phe represents a novel class of glycoprotein IIb/IIIa inhibitors, which act at the final common pathway of **platelet aggregation**. Although the specific role of RGD inhibition in the clinical setting remains undefined, a broad range of platelet-mediated primary and recurrent **thromboembolic** conditions may potentially benefit from therapeutic intervention with this compound.

AB . . . analogue, to inhibit human platelet function in vitro and to reduce in vivo canine platelet deposition on prosthetic grafts. Human **platelet aggregation** induced by 10 .mu.mol/L **adenosine** diphosphate was inhibited in a concentration dependent manner with an ED50 of 1 .mu.g/ml of SC-49992. **Adenosine** diphosphate-induced secretion, which is dependent on fibrinogen occupancy of the glycoprotein IIb/IIIa receptor, was reduced in a concentration dependent manner,. . . reduced by the analogue, but to a lesser extent. SC-49992 effectively eluted glycoprotein IIb/IIIa bound to RGD derivatized

sepharose. Eight **thrombosis**-prone dogs had polytetrafluoroethylene femoral artery grafts placed. Dogs received the RGD analogue or a normal saline infusion during their first. . . . or complete elimination of platelet thrombus on the luminal surface of the grafts under drug-treated conditions. Previous attempts to block **platelet aggregation** have been of limited success. 8-guanidino-octanoyl-Asp- Phe represents a novel class of glycoprotein IIb/IIIa inhibitors, which act at the final common pathway of **platelet aggregation**. Although the specific role of RGD inhibition in the clinical setting remains undefined, a broad range of platelet-mediated primary and recurrent **thromboembolic** conditions may potentially benefit from therapeutic intervention with this compound.

=>

L10 ANSWER 4 OF 9 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 96038544 MEDLINE
DOCUMENT NUMBER: 96038544 PubMed ID: 7502270
TITLE: Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection).
AUTHOR: Armstrong J K; Meiselman H J; Fisher T C
CORPORATE SOURCE: Department of Physiology and Biophysics, University of Southern California, School of Medicine, Los Angeles 90033, USA.
CONTRACT NUMBER: HL 41341 (NHLBI)
HL 48484 (NHLBI)
HL15722 (NHLBI)
SOURCE: THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.
Journal code: 0326377. ISSN: 0049-3848.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199601
ENTRY DATE: Entered STN: 19960217
Last Updated on STN: 19970203
Entered Medline: 19960118

AB RheothRx Injection, an aqueous solution of a nonionic block copolymer (poloxamer 188) formulated for intravenous administration, was investigated as an inhibitor of red blood cell (RBC)-induced **platelet aggregation** at plasma concentrations of 0.05-5mgmL-1. **Platelet aggregation** was determined by measuring the fall in single platelet counts after mechanical agitation of 2mL aliquots of citrated whole blood in a 37 degrees C shaking waterbath. Inhibition of RBC-induced **platelet aggregation** of > 95% was observed for poloxamer 188 at a concentration of 1mgmL-1, and 41% inhibition was observed at 0.05mgmL-1. Poloxamer 188 was observed to be a more effective inhibitor of RBC-induced **platelet aggregation** than 2-chloradenosine (2-ClAd) or phosphoenolpyruvate/pyruvate kinase (PEP/PK). Studies using platelet rich plasma (PRP) showed that **platelet aggregation** could not be induced by shaking in the absence of RBC, though aggregation was induced by the addition of **exogenous adenosine** diphosphate (ADP). Poloxamer 188 did not inhibit ADP-induced **platelet aggregation**. We propose that poloxamer 188 protects RBC from mechanical trauma by non-specific adsorption of copolymer to the RBC surface (via the hydrophobic polyoxypropylene moiety), and that this effect prevents mechanical damage and hence leakage of ADP from RBC. RheothRx Injection has been shown to have value in the treatment of acute ischemic disorders such as myocardial infarction. The observation of significant inhibition of RBC-induced **platelet aggregation** at clinically relevant concentrations suggests that RheothRx Injection may have antithrombotic properties in vivo, and may therefore have potential not only in acute ischemia but also to prevent thrombosis within vascular prostheses or to prevent rethrombosis after angioplasty or endarterectomy.

L10 ANSWER 4 OF 9 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 96038544 MEDLINE
DOCUMENT NUMBER: 96038544 PubMed ID: 7502270
TITLE: Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection).
AUTHOR: Armstrong J K; Meiselman H J; Fisher T C
CORPORATE SOURCE: Department of Physiology and Biophysics, University of Southern California, School of Medicine, Los Angeles 90033, USA.
CONTRACT NUMBER: HL 41341 (NHLBI)
HL 48484 (NHLBI)
HL15722 (NHLBI)
SOURCE: THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.
Journal code: 0326377. ISSN: 0049-3848.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199601
ENTRY DATE: Entered STN: 19960217
Last Updated on STN: 19970203
Entered Medline: 19960118

AB RheothRx Injection, an aqueous solution of a nonionic block copolymer (poloxamer 188) formulated for intravenous administration, was investigated as an inhibitor of red blood cell (RBC)-induced **platelet aggregation** at plasma concentrations of 0.05-5mgmL-1. **Platelet aggregation** was determined by measuring the fall in single platelet counts after mechanical agitation of 2mL aliquots of citrated whole blood in a 37 degrees C shaking waterbath. Inhibition of RBC-induced **platelet aggregation** of > 95% was observed for poloxamer 188 at a concentration of 1mgmL-1, and 41% inhibition was observed at 0.05mgmL-1. Poloxamer 188 was observed to be a more effective inhibitor of RBC-induced **platelet aggregation** than 2-chloradenosine (2-ClAd) or phosphoenolpyruvate/pyruvate kinase (PEP/PK). Studies using platelet rich plasma (PRP) showed that **platelet aggregation** could not be induced by shaking in the absence of RBC, though aggregation was induced by the addition of **exogenous adenosine** diphosphate (ADP). Poloxamer 188 did not inhibit ADP-induced **platelet aggregation**. We propose that poloxamer 188 protects RBC from mechanical trauma by non-specific adsorption of copolymer to the RBC surface (via the hydrophobic polyoxypropylene moiety), and that this effect prevents mechanical damage and hence leakage of ADP from RBC. RheothRx Injection has been shown to have value in the treatment of acute ischemic disorders such as myocardial infarction. The observation of significant inhibition of RBC-induced **platelet aggregation** at clinically relevant concentrations suggests that RheothRx Injection may have antithrombotic properties in vivo, and may therefore have potential not only in acute ischemia but also to prevent thrombosis within vascular prostheses or to prevent rethrombosis after angioplasty or endarterectomy.

L10 ANSWER 5 OF 9 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1993:517406 BIOSIS
DOCUMENT NUMBER: PREV199345116031
TITLE: Endogenous adenosine, but not **exogenous adenosine**, inhibits **platelet aggregation** and cyclic flow variations in dogs with coronary artery stenoses and endothelial injury.
AUTHOR(S): Nakayama, Hiroshi; Ikeda, Hisao; Oda, Tameo; Kuwano, Kazunori; Ueno, Takafumi; Koga, Yoshinori; Toshima, Hironori
CORPORATE SOURCE: Third Dep. Intern. Med. Kurume Univ. Sch. Med. Japan
SOURCE: Japanese Circulation Journal, (1993) Vol. 57, No. 7, pp. 675.
Meeting Info.: 57th Annual Scientific Meeting of the Japanese Circulation Society Kyoto, Japan March 25-27, 1993
ISSN: 0047-1828.
DOCUMENT TYPE: Conference
LANGUAGE: English

TI Endogenous adenosine, but not **exogenous adenosine**, inhibits **platelet aggregation** and cyclic flow variations in dogs with coronary artery stenoses and endothelial injury.

L10 ANSWER 6 OF 9 SCISEARCH COPYRIGHT 2003 ISI (R)
ACCESSION NUMBER: 92:163552 SCISEARCH
THE GENUINE ARTICLE: HG541
TITLE: PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA
AUTHOR: ELY S W (Reprint); BERNE R M
CORPORATE SOURCE: UNIV VIRGINIA, MED CTR, DEPT PHYSIOL, BOX 1116 MR4 ANNEX, CHARLOTTESVILLE, VA, 22908 (Reprint)
COUNTRY OF AUTHOR: USA
SOURCE: CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904. ISSN: 0009-7322.
DOCUMENT TYPE: Article; Journal
FILE SEGMENT: LIFE; CLIN
LANGUAGE: ENGLISH
REFERENCE COUNT: 199
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Endogenous adenosine, released from the heart in response to a decrease in the oxygen supply/demand ratio, and pharmacological concentrations of **exogenous adenosine** have protective effects in the ischemic myocardium. Adenosine is effective in limiting regional and global reperfusion injury and infarct size and appears to play a role in the phenomenon of preconditioning. The cardioprotective actions of adenosine are derived from several mechanisms: 1) coronary and collateral vessel vasodilation (increase in O2 supply), 2) negative inotropism, chronotropism, and dromotropism (decrease in O2 demand), 3) enhanced glycolytic flux and purine salvage, 4) reduction in microvascular injury during postischemic reperfusion by direct effect on the endothelium and by inhibition of neutrophil release of superoxide anions and **platelet aggregation**, and 5) possibly by stimulation of angiogenesis in states of chronic hypoxia or ischemia. The use of adenosine agonists may provide new methods for 1) preconditioning, 2) myocardial protection after thrombolytic or angioplastic recanalization of occluded or stenotic coronary arteries, 3) cardioplegic arrest, and, 4) organ preservation for cardiac transplantation.

L10 ANSWER 6 OF 9 SCISEARCH COPYRIGHT 2003 ISI (R)
ACCESSION NUMBER: 92:163552 SCISEARCH
THE GENUINE ARTICLE: HG541
TITLE: PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA
AUTHOR: ELY S W (Reprint); BERNE R M
CORPORATE SOURCE: UNIV VIRGINIA, MED CTR, DEPT PHYSIOL, BOX 1116 MR4 ANNEX, CHARLOTTESVILLE, VA, 22908 (Reprint)
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SOURCE: CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904. ISSN: 0009-7322.
DOCUMENT TYPE: Article; Journal
FILE SEGMENT: LIFE; CLIN
LANGUAGE: ENGLISH
REFERENCE COUNT: 199

ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Endogenous adenosine, released from the heart in response to a decrease in the oxygen supply/demand ratio, and pharmacological concentrations of **exogenous adenosine** have protective effects in the ischemic myocardium. Adenosine is effective in limiting regional and global reperfusion injury and infarct size and appears to play a role in the phenomenon of preconditioning. The cardioprotective actions of adenosine are derived from several mechanisms: 1) coronary and collateral vessel vasodilation (increase in O2 supply), 2) negative inotropism, chronotropism, and dromotropism (decrease in O2 demand), 3) enhanced glycolytic flux and purine salvage, 4) reduction in microvascular injury during postischemic reperfusion by direct effect on the endothelium and by inhibition of neutrophil release of superoxide anions and **platelet aggregation**, and 5) possibly by stimulation of angiogenesis in states of chronic hypoxia or ischemia. The use of adenosine agonists may provide new methods for 1) preconditioning, 2) myocardial protection after thrombolytic or angioplastic recanalization of occluded or stenotic coronary arteries, 3) cardioplegic arrest, and, 4) organ preservation for cardiac transplantation.

L10 ANSWER 8 OF 9 EMBASE COPYRIGHT 2003 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 83039773 EMBASE
DOCUMENT NUMBER: 1983039773
TITLE: Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin, and phospholipase.
AUTHOR: Bastida E.; Ordinas A.; Giardina S.L.; Jamieson G.A.
CORPORATE SOURCE: Am. Red Cross Blood Serv., Lab., Bethesda, MD 20814, United States
SOURCE: Cancer Research, (1982) 42/11 (4348-4352).
CODEN: CNREA8
COUNTRY: United States
DOCUMENT TYPE: Journal
FILE SEGMENT: 037 Drug Literature Index
029 Clinical Biochemistry
016 Cancer
025 Hematology
LANGUAGE: English

AB Three different mechanisms have been detected for the aggregation of platelets by tumor cells in a homologous human system based on inhibition studies with apyrase, hirudin, and phospholipase D. In the major group, **platelet aggregation** induced by the SKBR3 (adenocarcinoma), SKNMC (neuroblastoma), HT29 (adenocarcinoma), and HT144 (melanoma) cell lines was inhibited by apyrase and phospholipase D but not by hirudin, suggesting that adenosine 5'-diphosphate is involved in the first step. However, since the reaction occurs only in heparinized plasma, the mechanism must differ from that of **platelet aggregation** which can be induced in citrated platelet-rich plasma by endogenous or **exogenous adenosine** 5'-diphosphate. In contrast, the Hut28 (mesothelioma) line was inhibited by hirudin and phospholipase D but not by apyrase, suggesting that the mechanism in this system involves the activation of the clotting system in the early stages. However, the coagulant-dependent mechanism observed with Hut28 can be differentiated from the similar mechanism we have observed previously with the U87MG (glioblastoma) cell line since the latter is unaffected by phospholipase D (Am. J. Hematol., 11: 367-378, 1981). Phospholipase C had no effect on **platelet aggregation** induced by any of the human cell lines examined while both phospholipase A2 and lysolecithin inhibited aggregation in every case. These results suggest that two categories of human tumor cells can be defined based on whether they initiate **platelet aggregation** by adenosine 5'-diphosphate or coagulant-dependent mechanisms. However, within this latter category, subclassification is possible based on the inhibitory effects of phospholipase D.

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L9 ANSWER 44 OF 107 MEDLINE DUPLICATE 15

ACCESSION NUMBER: 92035426 MEDLINE
DOCUMENT NUMBER: 92035426 PubMed ID: 1657446
TITLE: Endogenous **adenosine inhibits platelet aggregation** during myocardial ischemia in dogs.
AUTHOR: Kitakaze M; Hori M; Sato H; Takashima S; Inoue M; Kitabatake A; Kamada T
CORPORATE SOURCE: First Department of Medicine, Osaka University School of Medicine, Japan.
SOURCE: CIRCULATION RESEARCH, (1991 Nov) 69 (5) 1402-8. Journal code: 0047103. ISSN: 0009-7330.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199111
ENTRY DATE: Entered STN: 19920124
Last Updated on STN: 19920124
Entered Medline: 19911127

AB The goal of this study was to clarify that blockade of adenosine receptors during myocardial ischemia causes further reductions in coronary blood flow due to platelet aggregation. Coronary perfusion pressure in 47 open-chest dogs was reduced such that coronary blood flow decreased to one fifth of the control value; thereafter, coronary perfusion pressure was maintained at the low levels. During hypoperfusion, coronary flow was kept low but constant with a massive release of adenosine. When 8-phenyltheophylline, an adenosine receptor antagonist, was infused during coronary hypoperfusion, coronary blood flow (18 +/- 2 ml/100 g/min) gradually decreased at 5-10 minutes of ischemia and reached almost zero at 20 minutes. Three minutes after the onset of ischemia, before further reduction of coronary flow, the microscopic examination revealed the existence of thromboembolization in the small coronary arteries, and the number of platelets in the regional coronary venous blood were significantly decreased, indicating that a further reduction of coronary flow due to treatment with 8-phenyltheophylline is attributed to thromboembolism caused by platelet aggregations. This reduction of coronary flow and formation of thromboembolism were inhibited by the treatments with dibutyryl cAMP, forskolin, and yohimbine, indicating that this thromboembolization during a lack of adenosine activity is due to platelet aggregation and that platelet aggregation caused by 8-phenyltheophylline is triggered by stimulation of alpha 2-adrenoceptors by released norepinephrine during ischemia. We demonstrate that adenosine, generated endogenously in response to ischemia, **inhibits platelet aggregation**. The finding that **adenosine** is not merely a vasodilator but that it also regulates thrombosis has major implications for designing new strategies of myocardial salvage.

TI Endogenous **adenosine inhibits platelet aggregation** during myocardial ischemia in dogs.

L22 ANSWER 3 OF 4 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1995:193637 BIOSIS
DOCUMENT NUMBER: PREV199598207937
TITLE: **Exogenous adenosine** application inhibits thrombus formation in stenosed canine coronary artery and partially protects against renewal of thrombus formation by epinephrine.
AUTHOR(S): Wang, T.; Lavis, J.; Bakalyar, D. M.; Catlin, T. R.; Timmis, G. C.; O'Neill, W. W.
CORPORATE SOURCE: William Beaumont Hosp., Royal Oak, MI 48073 USA
SOURCE: FASEB Journal, (1995) Vol. 9, No. 3, pp. A322.
Meeting Info.: Experimental Biology 95, Part I Atlanta, Georgia, USA April 9-13, 1995
ISSN: 0892-6638.
DOCUMENT TYPE: Conference
LANGUAGE: English
TI **Exogenous adenosine** application inhibits thrombus formation in stenosed canine coronary artery and partially protects against renewal of thrombus formation by epinephrine.
IT Miscellaneous Descriptors
ADENOSINE; ANTICOAGULANT-DRUG; ARTERIAL **THROMBOSIS**; CYCLIC FLOW REDUCTION; LEFT CIRCUMFLEX CORONARY ARTERY; MEETING ABSTRACT; STENOSIS

Gabel, Gailene

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1) Rubin et al., New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. Journal of Vascular Surgery, (1992) 15/4 (683-692).

2) Armstrong et al., Inhibition of red blood cell-induced platelet aggregation in whole blood by a nonionic surfactant, poloxamer 188 (RheothRx injection), THROMBOSIS RESEARCH, (1995 Sep 15) 79 (5-6) 437-50.

3) ELY et al., PROTECTIVE EFFECTS OF ADENOSINE IN MYOCARDIAL-ISCHEMIA, CIRCULATION, (MAR 1992) Vol. 85, No. 3, pp. 893-904.

4) Bastida et al., Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin, and phospholipase. Cancer Research, (1982) 42/11 (4348-4352).

5) Lee et al., In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency. BLOOD, (1979 Mar) 53 (3) 465-71.

6) Cattaneo et al., Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. BLOOD, (1990 Mar 1) 75 (5) 1081-6.

7) Wang et al., Exogenous adenosine application inhibits thrombus formation in stenosed canine coronary artery and partially protects against renewal of thrombus formation by epinephrine. FASEB Journal, (1995) Vol. 9, No. 3, pp. A322.

8)

STIC-ILL

BSI, P45

From: Gabel, Gailene
Sent: Monday, January 06, 2003 5:26 PM
To: STIC-ILL
Subject: 09/853,524

Please provide a copy of the following literature ASAP:

1) KUTSUNA et al., Identification and determination of platelet aggregation inhibitor from safflower (Carthamus tinctorius Linne). JOURNAL OF THE PHARMACEUTICAL SOCIETY OF JAPAN, (1988 Nov) 108 (11) 1101-3.

Thanks a bunch,
Gailene Gabel
7B15
305-0807

薬学雑誌
YAKUGAKU ZASSHI
108 (11) 1101—1103 (1988)

中国産紅花中の血小板凝集抑制物質の同定と定量

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(株)資生堂　基礎科学研究所,[a] 安全性・分析センター[b]

Identification and Determination of Platelet Aggregation Inhibitor from Safflower (*Carthamus tinctorius* LINNÉ)

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KAZUO KOMATSU,[b] and MOTOKIYO NAKANO[b]
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(Received April 21, 1988)

Safflower (*Carthamus tinctorius* LINNÉ) has been traditionally used for the treatment of Oketsu, namely blood stasis in Chinese medicine. Aqueous extract from safflower inhibited the rabbit platelet aggregation induced by adenosine diphosphate. The sole inhibitor was isolated by high performance liquid chromatography (HPLC), and identified as adenosine by ^{13}C- and ^{1}H-nuclear magnetic resonance. As the result of quantitative analysis by HPLC using the capsule type C_{18} column, 16—550 ppm of adenosine were detected in safflowers collected from different districts.

Keywords——safflower; *Carthamus tinctorius*; platelet aggregation; adenosine; C_{18} capsule type packing material

紅花 (*Carthamus tinctorius* LINNÉ; *Carthamus t.*) は，日本薬局方[1] 収載の生薬であり，主に駆瘀血薬，通経薬として血行障害や婦人病の治療に古くから用いられてきた．紅花は，末梢循環血流量の増加作用,[2] アドレナリンと拮抗する血管拡張作用,[1] ヒスタミンや抗血清と拮抗する血管透過性抑制作用,[3] 及び血液凝固抑制作用[4]を有することが薬理学的に確認されている．一方，紅花の成分は，赤色色素カルサミンと黄色色素サフラワーイエロー,[5] また変異原物質のケンフェロールとクェルセチン[6] の報告があるが，薬理作用の有効成分に関する報告はない．

著者らは，紅花の薬理作用について総合的に評価した結果，前述の薬理作用の他に血小板のアデノシン二リン酸 (ADP) による凝集に対し強い抑制作用を新たに見出した．そこで中国産紅花からこの血小板凝集抑制物質を単離，同定し，更に中国各産地の紅花中の血小板凝集抑制物質を定量したので報告する．

結　　果

(1)　血小板凝集抑制物質の単離及び同定

Chart 1 に示した分画のフローシートにおいて，紅花中の血小板凝集抑制物質は総て最終分画に濃縮され，この分画以外には血小板凝集抑制作用は全く認められなかった．単離した血小板凝集抑制物質を ^{1}H- 及び ^{13}C-核磁気共鳴 (NMR) スペクトルにより構造解析したところ，その構造はアデノシンと推定された．また，単離した血小板凝集抑制物質とアデノシン標品を瞬間マルチ測光検出器を用いた高速液体クロマトグラフィー (HPLC) により定性分析したところ，保持時間及び紫外部吸収スペクトル (λ_{max}=258 nm) が共に一致した．更に，単離した血小板凝集抑制物質は，アデノシン標品と血小板凝集抑制作用の強度が全く一致したことから，アデノシンであると同定した．

Carthamus tinctorius LINNÉ 20 g

extracted with H_2O (200 ml×3)
condensed to 30 ml
precipitated by addition of $(CH_3)_2CO$ 270 ml
dissolved ppt. in H_2O 30 ml
precipitated by addition of $(CH_3)_2CO$ 270 ml
evaporated in vacuum

filt. 1.04 g (50 μg/ml, 56%) | ppt. 4.85 g (250 μg/ml, 0%)

fractionated by HPLC
column: ODS (57 mm i.d.×300 mm)
eluent: AcOH : MeOH : H_2O = 0.1 : 25 : 74.9, 100 ml/min
evaporated in vacuum

420 mg (25 μg/ml, 53%)

fractionated by HPLC
column: ODS (20 mm i.d.×250 mm)
eluent: AcOH : CH_3CN : H_2O = 0.1 : 10 : 89.9, 10 ml/min
evaporated in vacuum

adenosine 4.2 mg (0.25 μg/ml, 43%)

Chart 1. Isolation of Adenosine

( ) indicates concentration and inhibition % of extent of aggregation.

(2) 血小板凝集抑制物質の定量

HPLCによるアデノシンの検量線は，0—30 μg の範囲で原点を通る直線 (相関係数 r=0.99990) を示した．紅花 2.0 g にアデノシン 1 mg を添加して回収実験 (n=6) を行ったところ，回収率 93.9%，変動係数 3.5% と良好な結果を得た．紅花水抽出物のクロマトグラム (Fig. 1) においてアデノシンは，約 4.5 分にシャープなピークとして溶出した．Table I に中国産紅花 (5 カ所) と山形産餅紅中のアデノシンの定量結果を示す．アデノシンは，16—550 ppm といずれの中国産紅花からも検出され，特に甘粛省産と新疆ウイグル自治区産のものの含有量が高かった．一方，山形産餅紅ではアデノシンは 5 ppm 以下で検出されなかった．

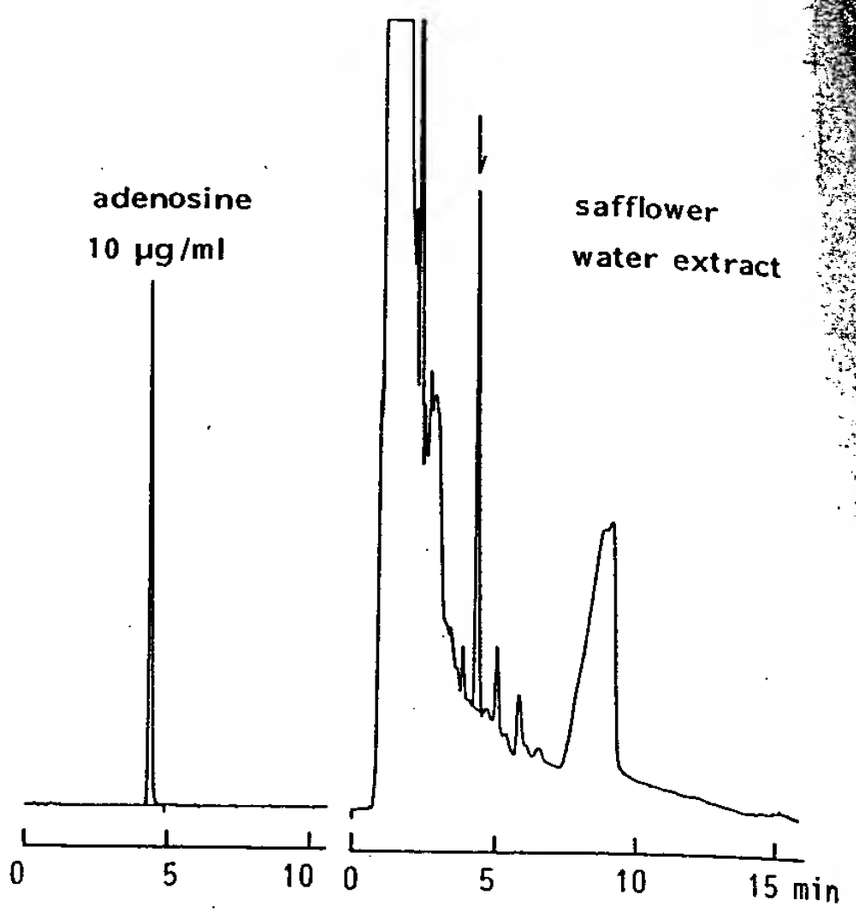

Fig. 1. Chromatograms of Adenosine and Safflower Water Extract

TABLE I. Analytical Result of Adenosine in Safflower

Sample	Harvest year	Adenosine (ppm)
Xinjiang Urygur	'85	518±58
	'86	550±71
Gansu	'85	522±49
Sichuan	'85	163±36
Yunnan	'85	139±24
Henan	'85	16± 5
Kyokuho	Unknown	336±45
Yamagata	'85	<5

n=5.

考　察

紅花の水抽出物が ADP による血小板凝集に対し強い抑制作用を有することを見出し，本作用成分をアデノシンと同定した．紅花水抽出物の血小板凝集抑制作用は，アデノシン以外の画分では全く認められないこと，また紅花水抽出物及び各画分の活性強度がそれらのアデノシン含有量に対応したことから，アデノシン単独による作用であるものと考えられる．アデノシンは従来より血小板の ADP による凝集を抑制することが知られているが，[8-10] 紅花中のアデノシンの存在を確認した報告は無い．今回の結果から，アデノシンが紅花の重要な有効成分の 1 つであることが確認された．しかしながら，紅花が有する血小板凝集抑制作用以外の多くの薬理作用についてはアデノシンのみでは説明できず，共存する他の有効成分に関する検討が更に必要である．

アデノシンは紅花以外にもサフラン，[11] 薤白及び大蒜[12]中にも血小板凝集抑制物質として存在することが報告されている．著者らによるアデノシンの分析法は，生薬中の未知の血小板凝集抑制物質を探索する際に，事前に

アデノシンの存在を確認する方法として有用であるものと考えられる．

実　験　の　部

試料　血小板凝集抑制物質の分画，単離には，新疆ウイグル自治区産紅花 (1985 年度産) を用いた．また，中国新疆ウイグル自治区産 (1985, 1986 年度産)，河南省産 (1985 年度産)，雲南省産 (1985 年度産)，四川省産 (1985 年度産)，甘粛省産紅花 (1985 年度産)，日本山形産餅紅 (1985 年度産)，及び局方紅花 (東京市場品) 中の血小板凝集抑制物質を定量した．

装置　分取——Waters 社 System 500A 分取専用 HPLC 装置及び Waters 社 HPLC 装置 (M600-U6K-M410-M740) を用いた．

定性，定量——Waters 社 HPLC 装置 (M600-M712-M400-M740) 及び大塚電子社瞬間マルチ測光検出器を用いた．

構造決定——日本電子社 JNM GX-400 型 ^{1}H-NMR 及び ^{13}C-NMR を用いた．

実験方法　血小板凝集抑制作用試験——日本白色種ウサギ (雄，体重 2.5—3.5 kg) の頸動脈あるいは耳介静脈から 3.8% クエン酸ナトリウムを抗凝固剤として常法通り採血した．採取した血液を 15 分間遠心分離し，上層より多血小板血漿を得た．血小板凝集はこの多血小板血漿及び凝集惹起剤として 2 μM ADP (シグマ社) を用いて，理科電気工業社製の凝集計により測定した．[7]

血小板凝集抑制物質の単離——新疆ウイグル自治区産紅花の乾燥花弁 20 g を水 200 ml, 70°C で 3 回抽出し，抽出液を合わせて 30 ml まで減圧濃縮した．これにアセトン 270 ml を添加して分別沈殿し，沈殿に水 30 ml を加えて再溶解しアセトン 270 ml を添加して再度分別沈殿し，上清を合わせて減圧乾固した (1.04 g)．抽出物を HPLC により Waters 社 ODS (57 mm i.d.×300 mm) カラムを用い，移動相；AcOH：MeOH：H_2O=0.1：25：74.9，流速；100 ml/min で粗分画した．更に YMC 社 ODS (20 mm i.d.×250 mm) カラムを用い，移動相；AcOH：CH_3CN：H_2O=0.1：10：89.9，流速；10 ml/min で分画を繰返すことによりアデノシン 4.2 mg を単離した (Chart 1)．

血小板凝集抑制物質の定量——各産地の紅花 2.0 g を水 40 ml, 70°C で 2 回抽出し，抽出液を合わせて水を加えて 100 ml に定容として，HPLC 用の試験液とした．HPLC の分析条件は，カラム；Shiseido Capcell Pak C_{18} (4.6 mm i.d.×250 mm)，カラム温度；40°C，移動相；CH_3CN：H_2O=5：95，流速；2.0 ml/min，検出波長；254 nm，試料導入量；10 μl とした．

アデノシンの核磁気共鳴スペクトル——^{1}H-NMR (in D_2O) δ: 3.94 (2H, dd, 5′-H), 4.36 (1H, s, 4′-H), 4.50 (1H, s, 3′-H), 4.85 (1H, s, 2′-H), 6.13 (1H, s, 1′-H), 8.31 (1H, s, 8-H), 8.39 (1H, s, 2-H). ^{13}C-NMR (in D_2O) δ: 62.3 (t, 5′-C), 71.4 (d, 2′-C), 74.5 (d, 3′-C), 86.6 (d, 4′-C), 89.2 (d, 1′-C), 119.8 (s, 5-C), 141.3 (d, 8-C), 149.1 (s, 4-C), 153.2 (d, 2-C), 156.3 (s, 6-C).

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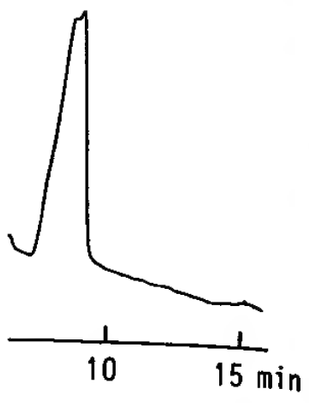
12) 奥山　徹，藤田賢司，柴田承二，保尊正幸，川田忠典，正木久朗，野口輝彦，日本薬学会第 108 年会講演要旨集，広島，1988 年 4 月，p. 292.

safflower
water extract



denosine and Saf-

: of Adenosine

ar	Adenosine (ppm)
	518±58
	550±71
	522±49
	163±36
	139±24
	16± 5
	336±45
	<5

pm 以下で検出さ

用成分をアデノシ
れないこと，また
シン単独による作
とが知られている
花の重要な有効成
くの薬理作用につ

在することが報告
する際に，事前に